CFTRI-MYSORE
982
Tissue culture t

4c2

499

TISSUE CULTURE TECHNIQUE

TISSUE CULTURE TECHNIQUE

By GLADYS CAMERON
Department of Biology
Washington Square College, New York University

SECOND EDITION
Revised and Enlarged

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
LIBRARY
25 OCT 1950
* MYSORE. *

1950
ACADEMIC PRESS INC., PUBLISHERS
NEW YORK

Copyright 1950, by

ACADEMIC PRESS INC.
125 East 23rd Street, New York 10, N. Y.

All Rights Reserved

No part of this book may be reproduced in any form, by photostat, microfilm, or any other means, without written permission from the publishers.

982

L,12

N50

PRINTED IN THE UNITED STATES OF AMERICA

CFTRI-MYSORE
982
Tissue culture t..

TO

PROFESSOR ROBERT CHAMBERS

IN AFFECTIONATE APPRECIATION OF HIS
INVALUABLE ADVICE AND STIMULATING ENTHUSIASM

FOREWORD TO THE SECOND EDITION

by

ROBERT CHAMBERS

The second edition of this book is most welcome. The first was universally commended and if simply reprinted would still have been well received. However, the keen interest of the author in her subject is displayed by the fact that, in the revision, every chapter has been checked and almost entirely rewritten. The author deserves great credit for the care she has taken not only in including modifications original with her but the results of a thorough search of the literature and consultation with many specialists in the field.

Although necessarily an increasingly involved subject the author has been able to retain the simple, direct and pleasingly conversational style which made the first edition such a success. The beginner will experience no difficulty in following the details of the technique but, what is more important, will find himself being irresistibly drawn more and more into the net of a fascinating field of research.

Being for so long an integral member of our Laboratory of Cellular Research Miss Cameron exhibits her intimate knowledge of cellular details, an essential part of tissue culture. During the academic year 1948-49 the author conducted a graduate course in tissue culture at New York University. She has made good use of her experience as is evident in her excellent planning and inclusion of features which may seem obvious to an advanced worker in the field but which are real stumbling blocks for the uninitiated. This book, which should cheer the heart of any beginner, does, at the same time, elucidate many details which have been sources of difficulty for the experienced worker.

The last chapter, written by C. G. Grand, also an integral member of our laboratory, is an excellent account of photomicrography as applied to living tissue cultures. In addition to his unquestioned research abilities in the cellular aspects of tissue culture, Mr. Grand has had many years' experience in photomicrography and has been extraordinarily successful in this most exacting branch of photographic technique.

New York, N. Y.
April, 1950

AUTHOR'S PREFACE

Acknowledgment is made to those investigators whose technical contributions are described, for having permitted the photographing of their special culture flasks and for the use of some of their illustrations. I also thank the graduate students, Mr. Leon Dziorney, Mr. Robert Kassel and Mr. Constantine Railey of the Biology Department, Washington Square College, for making some of the illustrations; and very specially Miss Louise Giraldi for her untiring efforts in the preparation of the manuscript. I am also much indebted to the staff of Academic Press for its cooperation and great assistance in handling the many burdensome details of publication.

I wish to express my sincere appreciation to my friend Dr. Jean Broadhurst, Professor Emeritus of Bacteriology of Teachers College, Columbia University for critical reading of the manuscript, and to Mr. Milton B. Wenger, Supervisor, Science Branches, Washington Square Library, New York University for his assistance in checking the references.

Special mention is made of my gratitude to Mr. C. G. Grand, Associate in this laboratory, for writing the chapter on photomicrography, for preparation of a large majority of the illustrations and for his skill and patience in the preparation of the photomicrographs and aid in their interpretation.

To Professor Chambers who has written the chapter on micromanipulation, I tender my sincere thanks. His life-long attitude and investigative spirit have instilled in his many students the stimulus expressed by Masefield,

> "Adventure on, for from the littlest clue
> Has come whatever worth man ever knew;
> The next to lighten all men by you . . ."

Gladys Cameron

Institute for Medical Research
Cedars of Lebanon Hospital
Los Angeles, Calif.

CONTENTS

PAGE

Foreword to the Second Edition, by Dr. Robert Chambers . . . vii

Author's Preface ix

CHAPTER

I. Introduction to Tissue Culture 1

II. Basic Considerations of Space and Equipment 5

Cleanliness, ventilation, lighting—One room, cubicle. Two or more rooms—Permanent equipment and its care.

III. Details of Equipment 11

Glassware, instruments, accessories—Amounts required—Cleansing and packing.

IV. Sterilizing Procedures 31

General precautions—Use of heat: dry heat, autoclave, Arnold—Filtration.

V. Physiological Solutions 34

Preparation of Tyrode solution, hydrogen ion concentration, osmotic pressure—Methods of sterilization: autoclave, filtration—Storage—Sterility tests—Formulae for various physiological solutions.

VI. Plasma and Serum 41

Preparation of paraffined tubes—Obtaining plasma of chickens: from carotid artery, wing, heart—Desiccated plasma—Use of anticoagulants—Obtaining blood of mammals—Obtaining blood of cold-blooded animals—Preparation of serum from plasma.

VII. Preparation of Tissue Extracts 54

Preparation of small amounts: chick, mammalian and amphibian embryonic extracts—Preparation of large amounts; use of mechanical devices—Desiccated extracts—Adult tissue extracts—Storage; freezing.

VIII. Artificial Media 59

Media of known composition—Fibrinogen and thrombin.

CHAPTER PAGE

IX. Preparation of Tissues 63

Warm blooded: embryonic, adult—normal and malignant—Cold blooded: vertebrate, invertebrate—Storage of tissues—Obtaining pure cell cultures.

X. Choice of Methods for Culturing 72

XI. Cultures on Coverslips 75

Preparation on single and double coverslips, renewal of medium, transfer—For growth, cytological study.

XII. Cultures in Flasks 85

Preparation in Carrel or Erlenmeyer, use of perforated cellophane, use of fibrinogen—Renewal of medium, transfer—For encouragement of differentiation, study of metabolic products of the cells, propagation of viruses.

XIII. Cultures in Roller Tubes 92

Preparation, renewal of medium, transfer—For study of metabolic products and nutritional requirements of cells, maintenance of strains of cells.

XIV. What One Sees in Tissue Cultures 96

Explants and outgrowths—Types of cells in outgrowths from various tissues—Dark field illumination and phase contrast microscopy.

XV. Some Experimental Procedures 116

Need for exactitude in preparation and selection of cultures, control and experimental—Keeping of records, cytological and physiological studies, growth measurements.

XVI. Micromanipulation of Tissue Cultures 122

By Robert Chambers

XVII. Histological Techniques 128

Vital staining—Fixation and staining—Sectioning.

XVIII. Photomicrography of Tissue Cultures 138

By C. G. Grand

Appendixes

A. List of Precautions to Insure Good Growth 166

B. Conversion Tables 168

C. Sources of Supply for Special Tissue Culture Materials . . 169

Bibliography 172

Supplementary References 181

Index 183

CHAPTER I

INTRODUCTION TO TISSUE CULTURE

The principles of tissue culture are easily understandable and the technique applicable to innumerable problems in biology and medicine. Modern methods stress simplification, which facilitates wider use with relatively inexpensive equipment and less space than has been hitherto possible. Nevertheless, experimentation by this method is not to be undertaken lightly. It requires not only technical skill, with extreme care in every step of the preparation and execution, but also knowledge of the normal cytology of the cultured material. The doubtful value of a considerable portion of the large volume of literature based on tissue culture is due less to faulty technical procedures than to lack of sufficient cytological training for accurate interpretation of the results. By following certain routines, cells may be made to grow and even give evidence of functional activity, but to what end if critical evaluation is lacking?

Tissue culture consists of removing tissues under sterile conditions from a living organism and incubating them in an environment (nutriment, aeration, temperature) conducive to growth. Variations in the technique have been developed in accordance with the type of cellular activity desired. The most successful methods deal with the multiplication of individual cells, and with the maintenance of cells in organized groups for a study of certain phases of their function.

An indispensable requirement for growth is that substances necessary for the life of the cell be in excess, so that their intake may be greater than their expenditure. When the nutritive material in the culture medium has been exhausted, fresh nutriment must be supplied to provide for more than mere maintenance of the integrity of the cells. The more active the tissue the greater is the wear and tear with a concomitant accumulation of waste products. Unless these are removed cellular activity is depressed, deterioration takes place, and eventually death ensues. True growth involves an increase in volume. The mere spreading of the tissue of an explanted fragment together with migration of cells already present in the explant may give a false appearance of growth. A true criterion is the actual increase in the size of the cells, and their subsequent proliferation.

Most of the tissues cultured have been those of warm blooded animals but the method has also been adapted to cold-blooded organisms including invertebrates, and to plant tissues. The technique for growing either cold- or warm-blooded animal tissues is basically the same. Plant tissue cultures, up to the present time, come under a somewhat different category being concerned largely with developmental processes rather than with unorganized growth. For the technique of plant tissue culture, the reader is referred to the pioneer work of Robbins (1922), and to others who have made extensive studies on the morphology and nutritional requirements of plant cells *in vitro,* for example, White (1942), Hildebrandt, Riker and Duggar (1946), Riker and Gutsche (1948), Galston (1948) and Caplin (1947). In the techniques thus far developed, the cells usually grow and proliferate in masses so that the criterion of growth has been mainly measurement of volume of the implanted fragments. For cytological study the tissues are sectioned.

Among the first to grow tissues *in vitro* was Leo Loeb (1897). Before him there had been fragmentary reports claiming the maintenance of living tissues in salt solutions or in body fluids. Loeb (1902) found that he could obtain cellular growth in clots of blood plasma or on sheets of agar which had been inserted under the epidermis of the rabbit's ear. Later, he demonstrated that cells could be kept alive and growing from explanted tissue fragments embedded in blood plasma in test tubes. The actual visualization of the growth of cells is to be credited to Harrison (1907) who observed under the microscope the migration of nerve fibers from fragments of the neural tube of a frog embryo implanted in frog lymph on a glass slide.

Burrows, as a medical student working in the laboratory of F. P. Mall at Johns Hopkins University, had already arrived at the idea of tissue culture by noting the growth from skin grafts separated from the edges of the wound by a space filled with exudate. From this observation he realized the important fact that growth was taking place independently of organic continuity with the body. He discussed his ideas with Harrison and continued the work of developing a medium similar to the exudate by planting fragments of chick embryo tissues on coverslips in a blood plasma clot. Burrows (1910) was the first to observe cells undergoing mitosis *in vitro.* He also noted that cells would grow only within the limits of their food supply. Cooperating with Carrel (1910), the foundations of the present coagulated medium technique were laid and adult mammalian tissues were successfully grown in blood plasma. A brief historical review of the subject up to 1912 is given by Burrows (1913).

A considerable advance was made in the technique when it was realized that, while coagulated plasma makes an excellent supporting framework for the cells in the culture, it does not contain the substances necessary for continued growth. Carrel (1912) found that the addition of aqueous extracts of embryos renders continuous proliferation possible. Evidence of this is found in his "old strain" of fibroblasts, which was maintained for over thirty years. Essentially this technique is the basis of many of the culture methods used today.

A variant of the plasma culture method was developed by Lewis and Lewis (1911b) and Lewis (1916), who pioneered in the use of fluid media. They used various combinations of salt solutions containing bicarbonate buffers and occasionally with the addition of peptone. They have also been successful with diluted sea water, serum, embryonic aqueous extracts and various combinations of these. The thin sheets of outgrowing cells make excellent preparations for cytological studies although with the fluid medium technique it has been difficult to maintain the cells at their optimum activity for longer than a few days.

In 1948 the Tissue Culture Association, working under a grant from the Committee on Growth of the National Research Council and the American Cancer Society, sponsored the commercial preparation of biological and synthetic media for tissue culture. This plan was initiated by the findings of Hetherington (1944) that media such as plasma and extract may be stored in a desiccated state without loss of activity. Lyophilized plasma, sera and extracts are now obtainable in small vials,* which, with the addition of a requisite amount of distilled water, provide materials for replacing media freshly prepared in the laboratory. The use of this standardized media is a long step forward and adds greatly to the uniformity of the basic material for making cultures. It is not only a time-saving factor but serves to eliminate some of the required equipment otherwise necessary.

There are certain basic principles, simple in themselves, that are essential for successful cultures irrespective of the culture method employed. These are: a) strict sterile precautions in regard to glassware, instruments, salt solutions and biological media, b) care in handling to prevent trauma and drying of the tissues to be cultured, and c) careful attention to the composition and hydrogen ion concentration of the medium, and d) to the temperature of incubation.

It is recognized in embryology that multiplication and organization of the cells do not proceed concomitantly. Since this principle applies also to tissue cultures the various techniques fall into two categories, those that promote cellular proliferation and those that enhance organization and functional activity. Growth necessitates frequent renewal of the culture medium while differentiation, on the other hand, requires avoidance of disturbance of the tissues, a limitation of growth stimulating substances, and in some cases lowered incubation temperatures.

---

* A list of the materials now available commercially is given in Appendix C.

In this book, an attempt has been made to reduce all equipment and procedure to their simplest forms without omitting steps necessary to ensure successful cultures. Sufficient detail is given to enable acquisition of the essentials of the techniques and avoidance of the many pitfalls which may be encountered by beginners, and may sometimes beset those more experienced. The prospective worker will naturally choose the methods most suitable for his problem and will doubtless introduce his own modifications. He is urged, however, to explore thoroughly a basic technique before he alters it, for each step is purposeful and has been carefully considered and tried.

The first few chapters of this book are devoted to the choice and organization of the laboratory rooms and their equipment, including glassware, instruments, etc. Attention is also given to methods of preparation of supplies for use in the various techniques. The succeeding chapters describe the preparation of the culture media and the tissues, as well as the preparation of the cultures in various ways. Also discussed are the types of cells one may expect to see growing from a given tissue, methods of recording their behavior and measuring their growth, as well as their significance in the interpretation of experimental results. The last chapters treat methods of applying micrurgical, histological and photomicrographic techniques to tissue cultures. At the end of the book appendices will be found listing possible sources of hard to find items used in tissue culture, also the ready-to-use constituents for culture media obtainable commercially and some conversion tables. It is hoped that the carefully considered data presented in this book, and the many details which are the result of long experience, may be of real service to the prospective worker.

CHAPTER II

BASIC CONSIDERATIONS OF SPACE AND EQUIPMENT

Adequate work in tissue culture does not demand the elaborate organization which for many years was considered necessary. A comparatively simple set-up is sufficient for use of the method for specific research problems as well as for supplemental use for diagnosis in pathological laboratories. Refinements and modern equipment have their advantages, but they are not essential. From the specifications of a desirable tissue culture unit each worker must select those which meet the exigencies of his own available space and equipment. Cultures can be and have been grown under far from ideal conditions. In the writer's opinion, one separate room is the minimum for satisfactory research work, and two rooms are a great advantage. Recently, we have found that a group of students in tissue culture technique working together in one room have been able to maintain sterile cultures for the duration of the course (four months). The culture table for each individual is protected by an open front lucite cover which has proved adequate for classroom work (Fig. 1).

The use of ultraviolet radiation is a simple inexpensive method for control of air-borne bacteria in the culture room. An appreciable reduction in the bacterial count (on plate cultures) is found where ultraviolet lamps are used for one half hour before commencement of culturing. They should not be used for any length of time while work is in progress because of possible deleterious effects on the tissues being cultured and to the operator.

Good light, cleanliness, freedom from air currents and careful preparation are all contributory to success; and each of these should be regarded as establishing a condition upon which the life of the cultures depends. The culture room should have smooth, clean walls and floor, with no unnecessary fixtures to collect dust. A uniform coat of grey or light green paint has been found to reduce glare and lessen eyestrain. A room with a skylight is ideal, but one with a good north window does very well.

As with all work done under sterile precautions, the problem of ventilation is difficult. Unless some artificial system is used, the room should be aired before and after work. While the work is in progress, the windows and doors must be kept closed.

Strict sterile precautions are always necessary and, obviously, problems concerned with long-term cultivation, where contaminations would nullify months of work, necessitate more stringent precautions than do those of short duration. For this purpose, if one room only is available, a section of it should be partitioned off to form a dust-proof *cubicle* for the sterile work. For one worker the size may be 5 × 6 feet although a larger one is more convenient and is necessary if two persons work together. The cubicle should be constructed of glass or glass bricks close to a window for daylight. Accessory light should be a fluorescent lamp outside and above the cubicle. A sliding door is preferred because drafts caused by opening and closing are thus greatly reduced. A sliding window with a platform sill on each side is a great convenience for passage of needed supplies into the cubicle. Easily cleaned flooring should be provided. Some laboratories use painted cement floors for the sterile room with drains to facilitate daily flushing. The writer prefers battleship linoleum which is also easily cleaned. Gas, electric and suction outlets should be provided as well as some type of ventilation without air currents. A system of air conditioning which cools, dehumidifies, filters and exchanges

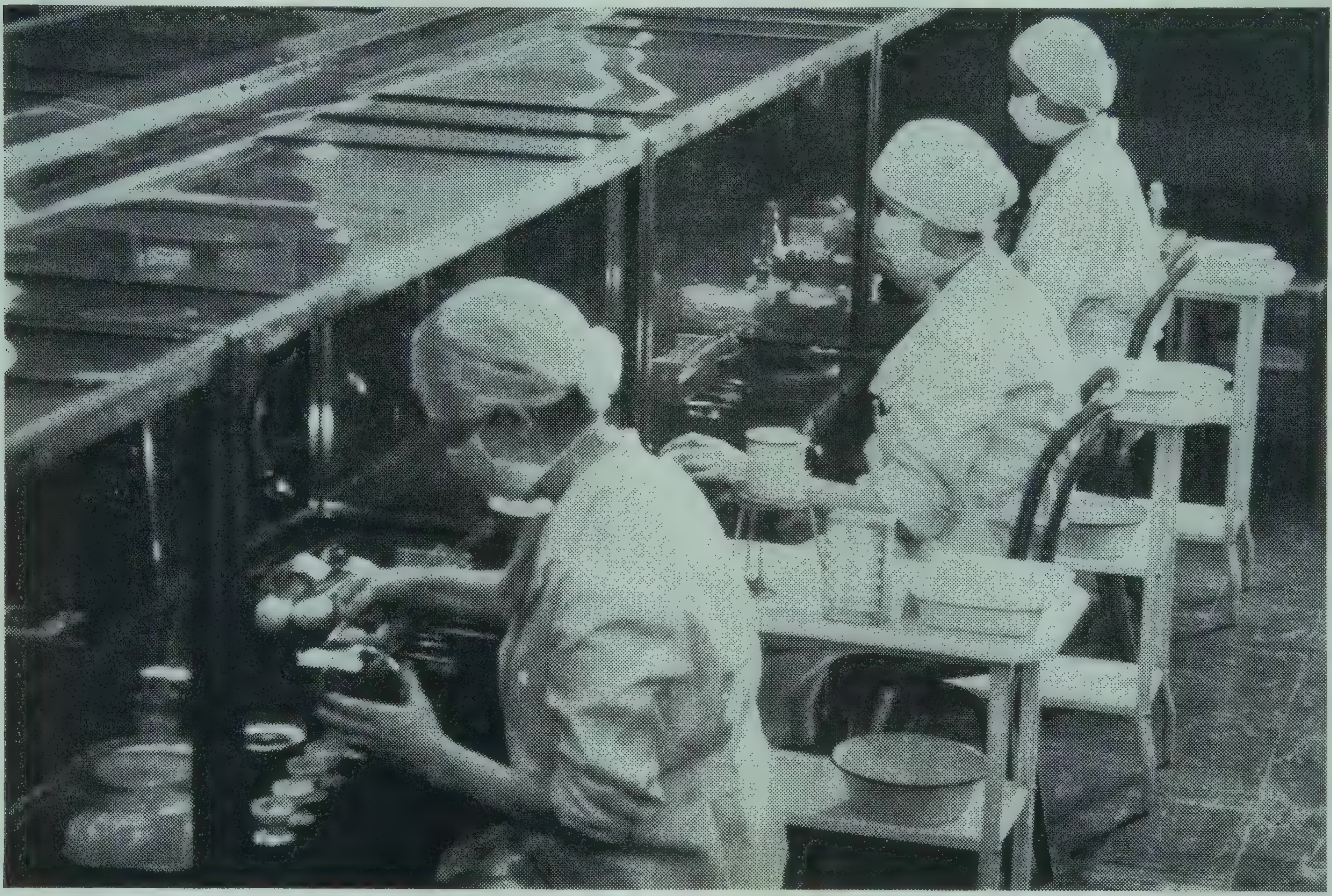


FIG. 1. Table arrangement for a group of students.

the air is recommended. Small units for this purpose are commercially available.

The culture table must have light, but no direct sun. Even a few moments of direct sunlight will injure most cultures. A removable sash of blue ground glass in sunny windows is helpful. It prevents injury to tissues, but is less restful to the worker's eyes than north light, or even that from east or west at those times when the sun is not directly on the window. Artificial light, subject to glare and interfering reflections and shadows, is less satisfactory than diffuse daylight, but lacking daylight, diffuse daylight bulbs may be used. When the table is in position, arrange the other equipment as conveniently as space will allow.

If space is not at a premium two rooms are much more convenient: a good sized preparation room from which opens a smaller culture room selected to obtain cleanliness and correct lighting. If possible, a combination scrub-up and dressing room through which the culture room is entered is an excellent addition, and an incubator room is also of advantage. Such a room serves several purposes. In it the revolving drum for the roller tubes may be placed, as well as shelves for slide cultures, trays for incubating eggs and an egg candling box. It may also solve the problem of suitable warm chambers for housing the microscopes for lengthy examinations of cultures and for photomicrography. This is accomplished by building extensions of the incubator room of a size suitable to accommodate a microscope with apertures on top for the ocular and on the sides. The worker remains in the outer room and inserts his arms through the side apertures to operate the microscope. The openings are covered with felt when not in use.

The equipment in the culture room (or culture cubicle) should be kept at a minimum as each added fixture increases the chance of dust contaminations. The following placement of equipment is recommended:

IN THE CULTURE ROOM

Culture table, assistant's table, microscope and table, culture incubator, closed cupboard for sterile supplies, cupboard for nonsterile supplies, wash basin, stools.

IN THE PREPARATION ROOM

Trip scale balance, centrifuge, autoclave, electric sterilizer, analytical balance, egg incubator, refrigerator, sink and drain board, stove, drying oven, work table, closed cupboards.

The size and shape of the *culture table* should be such that sufficient space is provided for efficient work. In our laboratory we use a table

4½ feet long by 3 feet wide, but some workers who have a permanent assistant prefer one of narrower width so that the assistant and operator may face each other across the table. In any case, the table should be firmly built and of such a height that the operator may work comfortably seated. Its position should be at right angles to the window, not directly facing it, and with the light coming from the left. This arrangement prevents shadows from the right hand from falling on the culture while under operation. Gas for a Bunsen burner, electricity for an electric plate and, if possible, suction for withdrawal of supernatant fluids from flask and tube cultures should be available.

The *assistant's table* for nonsterile accessories should be placed at the right of the culture table and at right angles to it.

The *microscope* placed on a table to the left of the culture table is convenient. For routine low-powered observation, a useful combination is a 10× objective and a 7× ocular. Higher magnifications for critical examination will also be necessary.

Culture incubator. In most commercial types the temperature varies somewhat in different parts of the incubator. The temperature of each shelf should be tested and use made of this apparent disadvantage when different types of tissue are to be cultivated. Mammalian tissues should be kept as near 37.5°C. as possible, but chick tissues grow well from 37.5 to 38° and are not killed at 39°C. An electric fan installed in the incubator will aid in maintaining an even temperature throughout, and a recording thermometer may be installed, although this is unnecessary in most cases. If roller tube as well as flask and slide cultures are to be made, unless an incubator room is available, a second incubator to house the rotating drum will also be necessary. Its size, of course, will depend on the number of cultures desired.

Balance. A trip scale is required for rough work, such as equalizing the weight of centrifuge tubes.

Centrifuge. A serviceable type has a carrier head for eight metal tubes and a speed up to 3000 revolutions per minute. About 2200 r.p.m. is desirable for obtaining plasma from blood and also for making the tissue extracts.

Autoclave. A horizontal type, 20 × 16 inches, heated by gas or electricity is serviceable.

Dry heat sterilizer. An electric type with thermoregulator is most desirable. The thermostat may be set at the desired temperature and left for the duration of the sterilization period. Gas may be used if necessary but is more difficult to regulate accurately.

Analytical balance. For weighing the ingredients of the salt solutions, etc., the balance should have a sensitivity of at least 1/10 mg. It should be placed on a firm, level table or shelf with minimum vibration. The Chainomatic type is accurate and saves time.

Egg incubator. This is used for obtaining chick embryos the tissues of which are frequently used for culturing and for making tissue extracts. A variety of models are on the market. A table model which will accommodate 60 eggs is obtainable. Others for larger numbers of eggs come with stands and utilize a motor and fan for air circulation. Unless carefully installed, vibration may affect the developing eggs by rupturing the blood vessels of the embryos.

Refrigerator. This should have a temperature control which can regulate the temperature to from 0 to 5°C. A temperature which fluctuates more than a few degrees causes deterioration of the ingredients of the culture medium. A small accessory refrigerator or cooler beside or under the culture table is very convenient.

Sink. This should be at least 30 × 20 × 12 inches with a shelf above it for a carboy of distilled water and a drain board of at least 30 × 20 inches with a drying rack above it for glassware, e.g., flasks, cylinders, etc.

Stove. A two or three burner stove should be placed close to the sink for boiling glassware.

Drying oven. The oven is used to dry glassware quickly.

The *worktable* should be as large as space will permit; 72 × 36 inches is convenient. On it much of the work of preparation will be done, viz., the glass slides and coverslips spread to dry, the cutting and folding of the gauze sponges, and the packing and wrapping of articles for sterilization.

A *closed cupboard* is essential for sterile supplies. Even though carefully packaged, all sterile supplies should be stored in closed cupboards because, when the wrappings are removed, dust on the coverings might fall onto the opened sterile materials.

A *cupboard* for nonsterile supplies may be the lower section of the above cupboard.

A *wash basin* for scrubbing the hands before culturing must be available.

The *stools* for use during the preparation of cultures should be of comfortable height to prevent fatigue. Stools are preferred to chairs whose backs may interfere with necessary sideway movements.

The egg incubator, refrigerator, trip scale balance, and centrifuge should be easily accessible to the culture room, since they are used while culturing. They might even form a part of the equipment in the culture room if the size of the rooms warrant it. It is well to remember, however, that each piece of equipment may accumulate dust.

CARE OF EQUIPMENT

As with all delicate work, the equipment must be kept clean and in good working condition. The temperature in the incubators and refrigerator should be checked daily, the centrifuge oiled from time to time and the brushes cleaned. The carrier cups should be removed and they, as well as the rubber cushions, dried after use. The heat control on the dry heat sterilizer should be checked against tested thermometers. Particular care must be given to the analytical balance. It is a delicate instrument of precision that will not stand rough treatment. It should be kept in a tightly fitting glass case, with a moistureproof cover to prevent dust or moisture from penetrating. The inside of the case and the balance must be kept scrupulously clean. Any traces of salts which may have been accidentally spilled should be removed with a camel's-hair brush. Needless to say, never put any chemicals directly on the pans. Weighing papers or balanced watch crystals must always be used. Weights must be handled with forceps. The box containing the weights must be tightly closed each time after use to prevent entrance of moisture from the air, the slightest trace of which may rust them. To maintain dry conditions a useful precaution is to keep some crystals of anhydrous $CaCl_2$ in an open dish in the glass case.

Doubtless anyone undertaking tissue culture is familiar with the use and care of the microscope. The various parts of the ocular system, oculars, objectives, condenser, etc., must be kept meticulously clean by frequent wiping with lens paper only. Never use harsh material for this purpose as harmful scratches may result. Immersion oil should never be allowed to dry on an objective and in examination of freshly stained preparations avoid smearing any trace of balsam on any part of the instrument. Except when in use, the microscope should be carefully covered with a moistureproof cover.

Chapter III

DETAILS OF EQUIPMENT

The equipment of a tissue culture laboratory includes a supply of many items of glassware and instruments which are used for other types of laboratory work. Certain specialized glassware and selected fine instruments are also required as well as various specially designed accessories. It is, of course, impossible and unnecessary to attempt to mention all the equipment and accessories used in the various tissue culture laboratories. Each worker has his own preferences and the requirements depend on the type of work being done. The following lists of glassware and instruments are those which have been found satisfactory for general tissue culture work.

GLASSWARE

All the glassware, both standard and special, should be new and of the best quality hard glass (such as Pyrex) with a minimum of free alkali. Once assigned for culture work, it must be used for no other purpose. Assuming that two persons are devoting full time to the work, a reasonable supply of glassware would be about as follows:

Standard Glassware

Flasks: Erlenmeyer	*Number*
2 liter	2
1 liter	2
500 cc.	4
250 cc.	12
Funnels	
100 mm.	2
Beakers	
Nest of 6 from 1 liter to 100 cc.	1 nest
Watch crystals	
115 mm. diam.	6

Special Glassware

Hollow ground slides	*Number*
75 × 25 mm. with round concavity	4 doz.
75 × 25 mm. with oval concavity	3 doz.
75 × 45 mm. with large round concavity	12 doz.
Coverslips	
22 × 40 mm. No. 1	3 oz.
22 mm. round No. 1	6 oz.
40 × 40 mm. mica	500 sheets
Graduated pipettes	2 doz.
Pipette tubing—hard glass	
About 7 mm. inside and 8 to 8.5 mm. outside diam.	3 lbs.

Graduated cylinders	
1 liter	1
500 cc.	1
100 cc.	1
50 cc.	1
25 cc.	1
10 cc.	1
Petri dishes	
100 mm.	2 doz.
150 mm.	6
200 mm.	6
Tubes (rimless)	
75 × 18 mm. (for blood)	6 doz.
100 × 25 mm. (for racks)	6 doz.
90 × 9 mm. (for extract)	3 doz.
15 × 100 mm. (roller tubes)	6 doz.

Solid glass tubing	
5 mm. in diameter	1 lb.
Pipette containers	
Large glass tubes 36 × 200 mm.	12
Boxes or moist chambers	
Square or round with covers. 100 × 200 mm. for packing slides	6
Carrel flasks	
5 cm. diameter	1 doz.
3 cm. diameter	2 doz.
Columbia staining dishes	3 doz.
Ampules	
1 cc. (for phenol red)	1 gross

SELECTION AND PREPARATION

Tissues removed from their natural environment are obviously at a disadvantage and, especially during the readjustment period in the culture medium, extremely sensitive to toxic substances with which they may come in contact. It is therefore essential that the greatest care be exercised in the preparation of the glassware, with special attention to that on which the cells are to be cultured (coverslips, flasks, tubes, etc.) and to the pipettes which introduce the culture medium. The task is arduous and there are no short cuts but, without proper attention to this basic step, there can be no hope of success in the growth or maintenance of healthy cells.

All the glassware should be boiled with soap in an agate pan, rinsed in running hot water, distilled water and alcohol and dried. Used glassware is rinsed in cold water before boiling.

Materials Required for Cleansing

Soft soap
Agate pans (1 or 2, covered, for boiling slides; 2 separate bowls for rinsing in distilled water and in alcohol; flat agate pans for boiling and rinsing pipettes; 1 or 2 small pans for boiling coverslips).
Distilled water
95% alcohol
Lint-free towels (linen glass towels or diaper cloth)
Wire baskets

The soft soap is prepared by boiling about 6 small cakes of Ivory soap in about 2 quarts of water. It may then be covered and used as needed. Soap powders should not be used unless a careful analysis indicates that they do not contain strong alkalis, ammonia or other harmful ingredients. Some workers prefer one of the newer detergents instead of soap and these may be used if desired. In this laboratory we have had better results with the use of a pure white soap such as Ivory or Fairy than with the detergents we have tested. However, in regions where the water is excessively hard, a detergent may prove more satisfactory than soap.

In certain types of experiments, such as those involving chemical analysis of the culture medium, the use of a chemical cleaning mixture may be necessary.

The cleaning mixture of sulfuric bichromate used by some workers for glassware, is prepared as follows:

Dissolve 40 g. of potassium bichromate in 1000 cc. of distilled water in a Pyrex flask. Pour slowly into this solution 80 cc. of concentrated C. P. sulfuric acid. Be careful that none is spilt as the acid will burn anything it touches.

This solution acts by virtue of its being a strong oxidizing agent and will remove adherent organic matter from glassware. It should be brick red in color. When it becomes green, it is reduced and as such it is useless. In order to prevent excessive oxidation and waste of the cleaning fluid, articles covered with soap, alcohol, etc., should be rinsed in water before they are placed in the cleaning solution.

The writer has found the use of cleaning fluid impractical and unnecessary in most cases. For slides, it is unnecessary. For coverslips, the risk of not getting rid of the solution even by repeated rinsing is greater than the possible advantage to be derived from it. However, it may be used with benefit for a few articles, e.g., new Erlenmeyer flasks of a design which is easily rinsed, Carrel flasks, when difficulty is encountered in entirely removing the plasma clot, and fine pipettes that are clogged with coagulated material. Whenever cleaning fluid is used, the glassware must be thoroughly rinsed afterward, using ammonia the first few times to neutralize the acid.

Coverslips

The selection and preparation of coverslips is of foremost importance. Those of the best quality hard glass, as well as being better for growing cells, are an economy in the end as loss from breakage is diminished. Thin sheets of mica, cut to any desired size, may be used to replace glass coverslips. They have an advantage over glass coverslips in being alkali-free and unbreakable. Their poor transparency, however, renders them less satisfactory for high power observation; and, in cutting cultures for transplants, the mica may also be cut thus roughening its surface and making proper cleaning difficult. Mica coverslips are particularly useful in the double coverslip culture method as the supporting large coverslip for the small circular glass one (Chapter XI). Cellophane, which is obtainable in any desired thickness, is sometimes used in place of glass coverslips. It is best sterilized by boiling. Its use is especially well adapted to sparsely growing tissues, such as those of plants, which are difficult to transplant successfully. Those sections of a culture on cellophane which contain growing cells may be cut out with a sharp knife or scissors and transferred to a fresh medium without disturbing the cells. The perforated cellophane used by Evans and Earle (1947) in their flask cultures may also be cut and used as coverslips. It should be cleaned in several changes of acetone, alcohol, ether and triple distilled water. It is then dried and autoclaved. The type, size and shape of the coverslips may vary, but not the care with which they are prepared. Carelessness in this process may nullify meticulous efforts elsewhere.

For washing coverslips. The larger coverslips and those made of mica may be boiled together. Those which have been previously used and have paraffin adhering to them should first be boiled separately to remove the paraffin and then added to the others for a second boiling. Place three or four dozen slips in a granite pan, cover with cold water, add a handful of soft soap, and boil for ten minutes. Then, holding at the edges between thumb and finger, rinse each one separately and thoroughly in running water. The water should be not merely warm but really hot in order to be sure that the soap is in solution and will not cake on the glass. As each one is rinsed, place it in a container of distilled water. Pour off the water and cover with 95% alcohol—which *has not been* previously used for *any* purpose. If the alcohol is poor in quality, it should be redistilled before using. Remove the coverslips from the alcohol with forceps. Hold at the edges only, between thumb and finger, and while drying with a lint-free towel, take care not to touch the surface. As each coverslip is dried, place it singly on another towel, making sure that each is thoroughly dry so that they will not stick together when placed in a Petri dish for sterilization.

The small round coverslips such as those used in the double coverslip method, should be prepared separately to prevent breakage. Since they will have no adherent paraffin they need one boiling only. The rinsing is done as described above. For drying the small thin circular coverslips, covered wooden blocks are convenient (Fig. 2). The blocks should be about 6 × 3 × $\frac{3}{4}$ inches, covered with a layer of tightly stretched silence cloth firmly fastened to the slides. One or two layers of lint-free material (handkerchief linen or nylon) are smoothed over the padding and fastened with thumb tacks. The linen is easily removed and should be washed and ironed after each time it is used. The coverslips are removed from the alcohol with forceps and placed in rows on one padded block, covered with the second block and the two rubbed lightly together. If the blocks are smooth, about two dozen coverslips will be dry, polished and intact in a few moments and may be readily shaken onto a clean towel or paper. This procedure saves time and coverslips since practically no breakage occurs. The coverslips are then placed in Petri dishes and wrapped in paper ready to be sterilized.

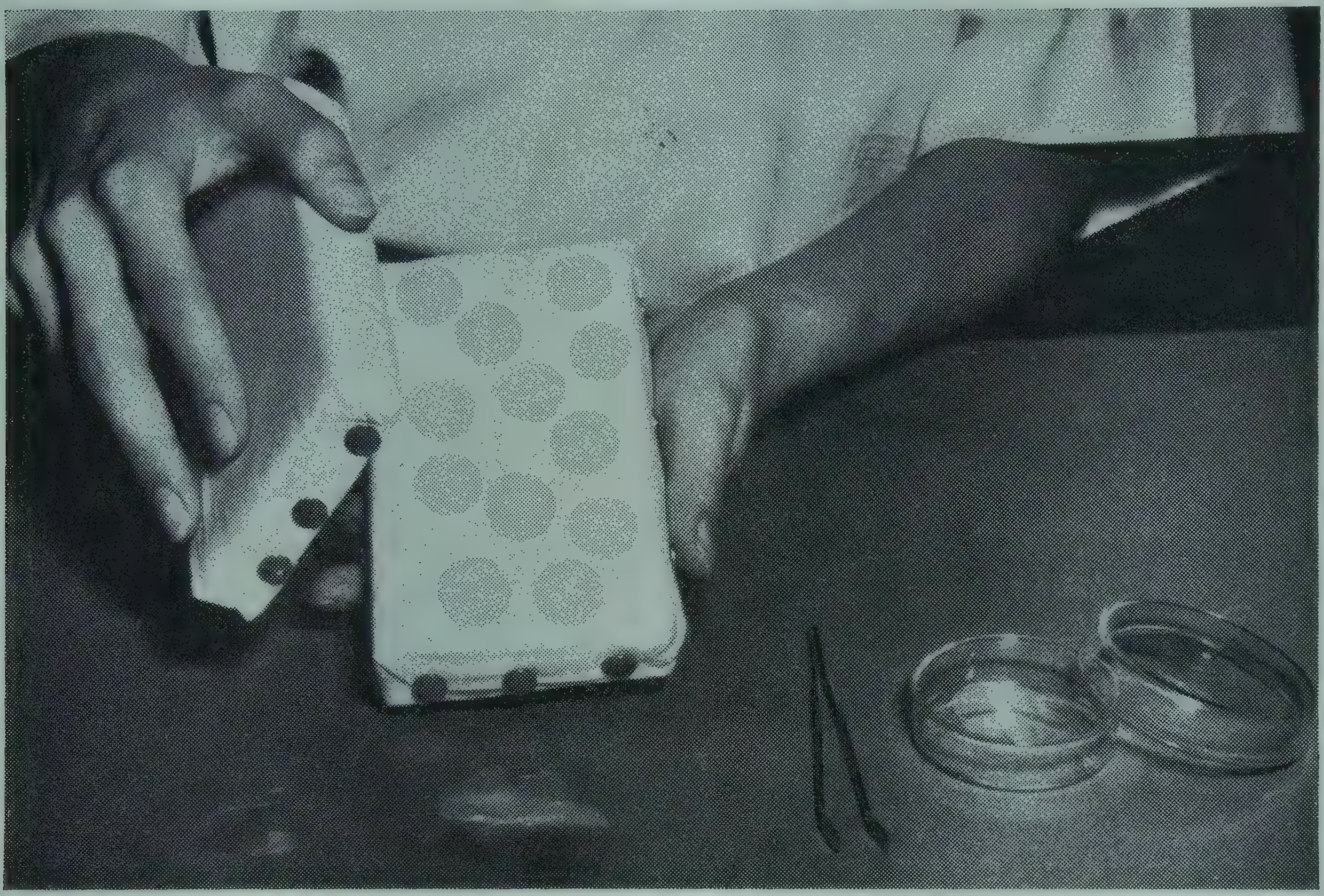


Fig. 2. Method for drying thin, small coverslips.

Pipettes

Pipettes of any desired size may be purchased, but the simple capillary type may be made less expensively in the laboratory with a little practice (Fig. 3).

Materials Required

Pipette tubing, hard glass, about 7 mm. inside, 8 to 8.5 mm. outside diameter
Bunsen burner
Fantail burner

Small file
Ruler

The two kinds of pipettes required for general use are fine ones, for placing media on coverslips and coarse ones for withdrawing plasma, physiological solutions, etc., and for preparing tissue extracts.

Fine or capillary pipettes. To prepare these, cut the tubing about 140 mm. in length. Use a blow pipe if necessary to obtain a fine point of blue flame from the Bunsen burner. Holding one end of the piece of tubing in each hand, constantly turn the central part in the flame until the glass softens. Then remove from the flame and pull evenly and quickly, and when cool, break at the middle. Two pipettes of about 1 cc. capacity are obtained and each should be about 140- to 150-mm. long, with the diameter at the narrow end about 1.5 mm.

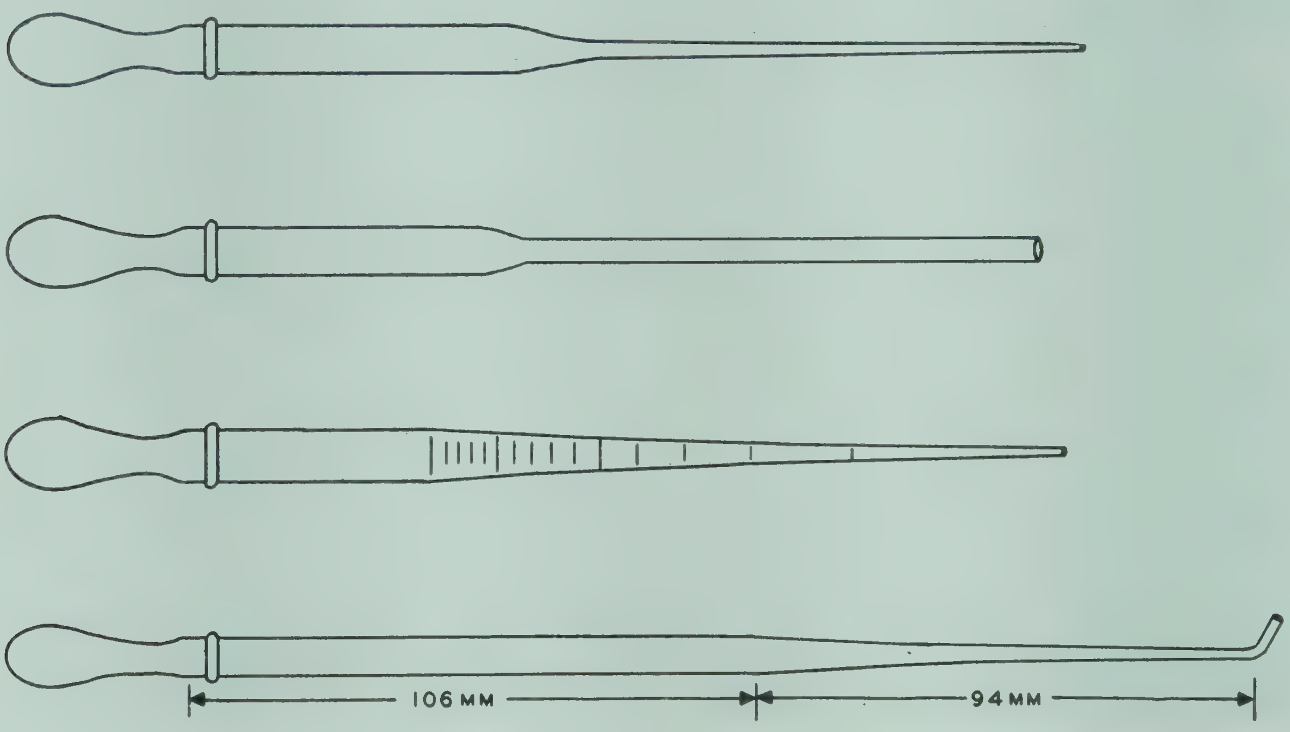


FIG. 3. Culture pipettes with nipples attached.

Coarse pipettes. Cut the tubing 150- to 160-mm. long, use the fantail burner, and pull out while still in the flame. The file will be needed to divide these pipettes smoothly, as the aperture at their points should be about 4 or 5 mm. in diameter and their length about 150 mm. with a capacity of 1 to 1.5 cc. Round off the edges of the upper end in the flame to prevent injury both to fingers and rubber nipples.

Bent tip pipettes. These are for use in preparing roller tube cultures. In cutting the tubing allowance should be made so that the length before bending the tip will be from 170 to 180 mm. A bend at an angle of about 45 degrees 5 mm. from the tip will leave the pipette at least 165-mm. long. This will reach to the bottom of the 150 mm. test tube without touching the top with the nipple. After the bend is made, the tip, about 2 mm. in diameter, must be carefully smoothed in the flame to prevent adherence of the tissue fragments to it during their placement in and removal from the tube.

Graduated pipettes for tissue culture are obtainable commercially and are a great convenience. It is difficult to make these accurately in the laboratory. They are graduated in tenths of a cubic centimeter, with a capacity of 1.5 cc. and a tip approximately 2 mm. in diameter. (See Appendix C.)

To prevent undue breakage of pipettes, they should not be washed with other glassware. We have found it helpful to have a flat oblong granite pan containing cold water placed on the assistant's table. As pipettes are used, they are discarded into the pan of water, which prevents drying of material or coagulation of media in them and thus simplifies subsequent cleansing. Later they are boiled with soap and go through the regular procedure of rinsing with hot running water, distilled water and alcohol.

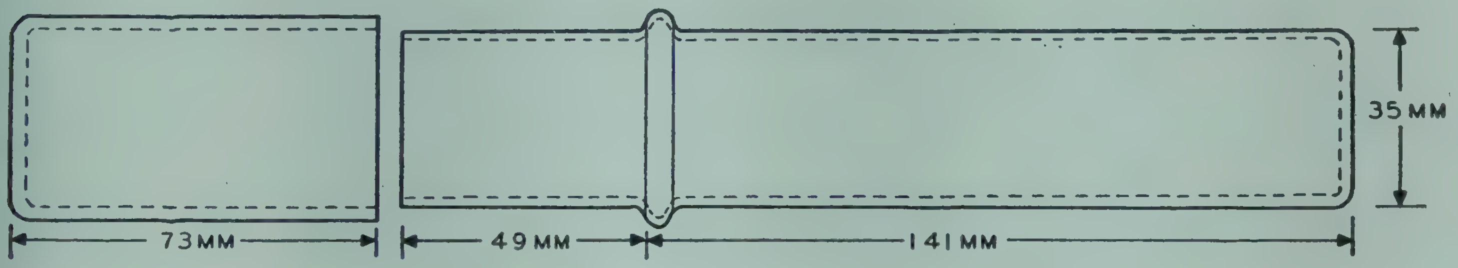


FIG. 4. Glass container for pipettes.

Special care must be taken that the solutions pass through the pipettes to ensure their being thoroughly rinsed *inside*. Place in a wire basket and dry thoroughly and quickly in a drying oven. The upper end is plugged with cotton and the pipettes placed points down in large glass tubes, with covers (Fig. 4). Tie a double layer of paper over the cover for sterilization. Of course metal containers may be used if desired but they are more expensive and it is advantageous to be able to select the desired pipette before withdrawing it from the sterile tube.

Carrel flasks must be given as much care as coverslips. When they are prepared for boiling, care must be taken that the cold soapy water fills the flasks. Thorough rinsing is essential. To ensure thorough rinsing, we have devised a monel metal drum (Fig. 5)—diameter 125 mm., depth 100 mm. Its central neck is connected with the hot water tap by rubber tubing; and the flasks, inverted over the smaller outlets around the periphery, are rinsed as water is forced through them. The flasks are filled with distilled water, and then with alcohol, after which they are turned bottom up to drain. When the flasks are dry, the necks are plugged with cotton, and the flasks placed in a glass or metal container and wrapped in paper prior to sterilization. We use discarded film tins for this purpose.

If the tap water is absolutely clear and distilled water is difficult to obtain in sufficiently large quantities, the latter may be omitted for everything but the coverslips, Carrel flasks, roller tubes and pipettes though such an omission is not to be recommended.

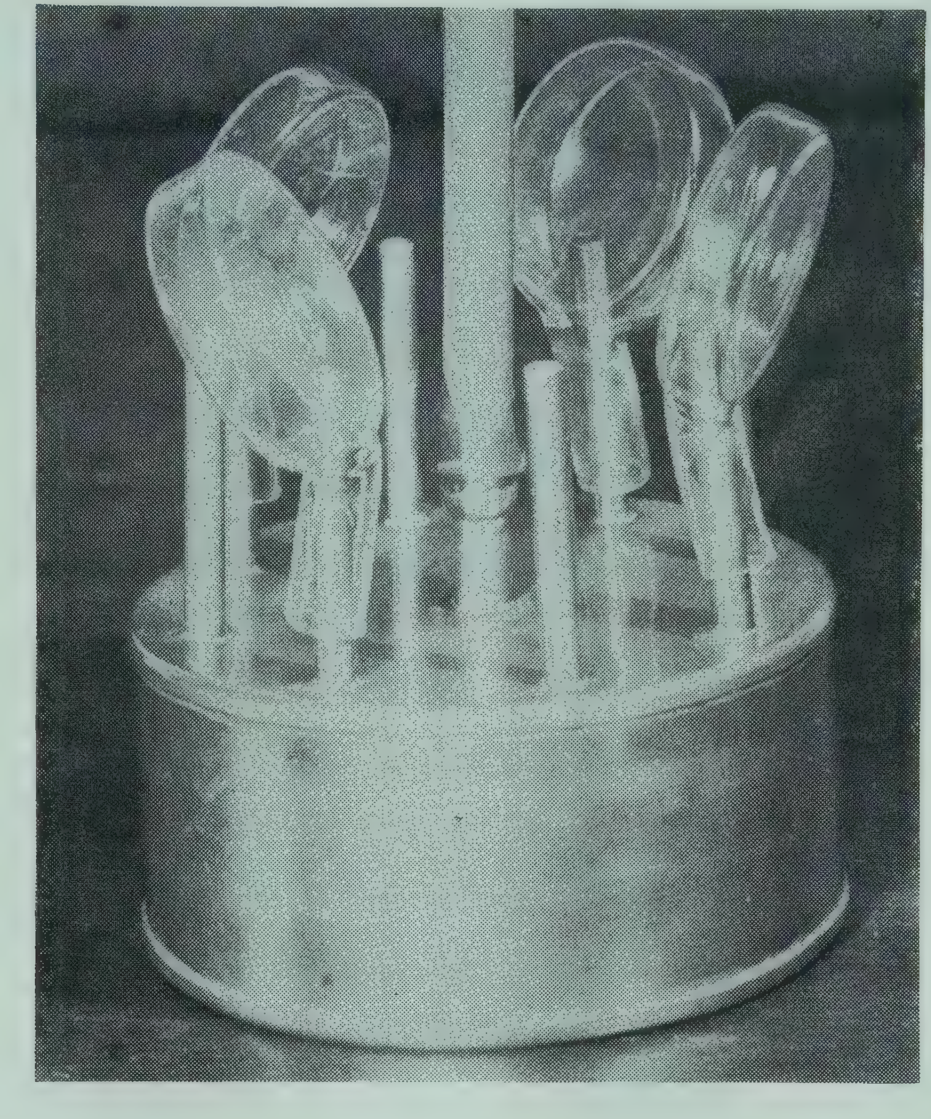


FIG. 5. Metal rinser with four flasks in place.

The remainder of the glassware such as tubes, slides, etc., may be boiled together. The tubes are inverted in a basket, dried in a dryer, corked and packaged in convenient numbers. Watch crystals, when dried, are placed concave side down in 150-mm. Petri dishes and wrapped in paper for sterilization. Depression slides are dried with lint-free towels and placed concave side down in a suitable container (we use "moist chambers"), so that even when the cover is removed each slide is protected from dust until used.

When the cleansing of the glassware is completed, the graniteware should be dried and the towels washed and carefully rinsed in the laboratory. They should not be sent to a commercial laundry where strong unknown chemicals may be used, traces of which may remain in the towels.

INSTRUMENTS

As a general rule the instruments required for tissue culture are those used for any fine surgical work such as eye surgery. Each type of work undertaken necessitates certain equipment the specific requirements for which will be taken up as each technique is described. In order to facilitate the assembly of a suitable supply, the following lists are offered as a guide.

Instruments for bleeding from the carotid artery

4 towel clamps	
4 hemostats	
2 knives (blades No. 22 and 23)	
3 small curved hemostats	115 mm.
1 very fine straight scissors	90 mm.
1 very fine curved iris forceps	100 mm.
1 very fine serrefine	25 mm.
1 scissors for silk	
1 No. 6 needle, curved cutting edge	
2 cannulas, sizes A and B	

For cultures

1 hemostat
6 coverslip forceps
6 cataract knives
12 dissecting needles
6 removable blades, No. 23

For embryo extract

1 fine straight scissors	100 mm.
1 curved iris forceps	100 mm.
1 curved on flat scissors	100 mm.

Miscellaneous

Needle holders	90 mm.
Platinum or platinum alloy wire (gauge 17)	150 mm.
Long curved sponge forceps	250 mm.
Platinum or platinum alloy hypodermic needle	50 mm.
Bandage scissors	

For Carotid Bleeding

Four hemostats. These are for general use, such as holding back the skin of the neck and for swabbing with a sponge; they may be of any size available.

Two knives. One knife is for the skin and one is for subcutaneous tissue. Bard Parker removable blades and handles are inexpensive and convenient, as they eliminate the necessity of sharpening. Sizes 22 and 23 are about right for the skin and subcutaneous tissue, respectively.

Three small curved hemostats (115 mm. length). One is for the blunt dissection of the blood vessel, and two for passing ligatures around it.

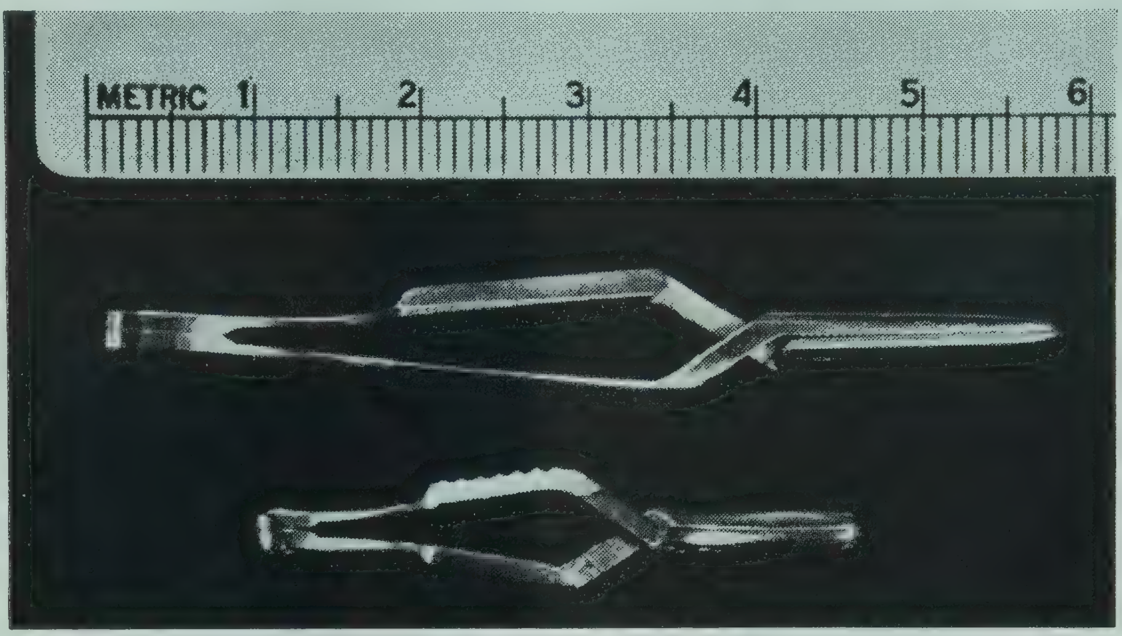


FIG. 6. Serrefines for controlling the flow of blood in an incised artery.

One straight fine scissors (90 mm. length). This must be fine and sharp for cutting the artery and should be used for no other purpose.

One small curved forceps (100 mm. length). For removing connective tissue from the dissected vessel and for exploring the incision in the artery.

The *small serrefine* (Fig. 6), the clamp used to control the flow of blood in an incised blood vessel, should be about 25 mm. in length. This is not always easy to obtain (recently we found it necessary to have some made), but it is almost a necessity when obtaining blood from the carotid artery.

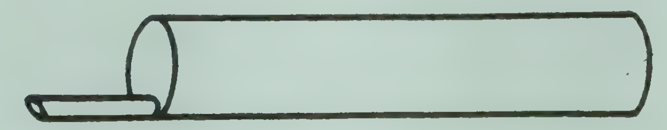


FIG. 7. Metal arterial cannula.

Arterial cannula (Fig. 7). One with a slender neck and beveled end is needed for easy insertion into the blood vessel. The silver-plated Porter cannula is obtainable in various sizes. Sizes A and B are good for chickens and small animals and, if sterilized and kept in olive oil, need not be paraffined. Glass cannulas may be used if desired, but the metal type is more easily handled and there is no danger of breakage when inserting or from accidental movements of the animal. Care must be taken to clean the cannula in cold water immediately after use, before the blood clots in it. It must then be rinsed in alcohol and thoroughly dried before being placed in the olive oil preparatory to sterilization.

Needle. It is desirable to have a needle with a cutting edge and sufficiently fine to prevent tearing the skin of fowls, which is thinner and more fragile than that of mammals. No. 6, curved, to be threaded with medium weight surgical silk, is a useful size.

Scissors. For cutting the silk, any small size will do.

For Embryo Extract

Straight scissors. For snipping the membranes surrounding the embryo.

Curved iris forceps. For lifting the embryo out of the egg.

Curved scissors. For cutting up the embryos in a watch crystal.

For Cultures

Hemostat. For lifting the pad covering the knives.

Coverslip forceps. One is needed for removing sterile coverslips from their Petri dish, the remainder for removing the small inner coverslip from the mica square when washing the double coverslip cultures.

Cataract knives with straight blades about 30 mm. in length are used for cutting the tissues and for making transplants. They are expensive but, if properly cared for, maintain their temper well even after repeated sterilizations. We have found scalpels with removable blades more clumsy, less firm and therefore less easily controlled. However, some workers find No. 7 blades (which have a straight cutting edge) quite satisfactory.

Miscellaneous

Platinum needle. May be used to hold the tissue when it is being cut (Earle uses one of tungsten) and, when heated in the flame, is useful for pricking any bubbles which may inadvertently have been introduced into the medium. If platinum is not available, nichrome wire will serve for removing the bubbles. The needle is made with a 25-mm. length of 17 gauge platinum or platinum alloy wire. The needle should be smoothed on a stone and mounted in a holder. Platinum wire is advised since it may be repeatedly sterilized by flaming, thus obviating the necessity for a large supply of sterilized dissecting needles. If the latter are used, they must be changed frequently because carbon deposits form on them when flamed, and these may fall into the culture.

Platinum spatula. Used for introducing tissues into Carrel flasks and for removal of the clot and tissues when transferring them. It is made with a 125-mm. length of 17-gauge platinum or irradium platinum wire; one end is hammered out to make the blade about 15-mm. long and 2- to 3-mm. wide; the blade must be smoothed on a stone and sandpapered, so that the tissue will not stick to it; the other end is inserted in a needle holder. This spatula is very convenient because it is pliable and may be bent into any desired shape, after which it is sterilized by flaming.

Platinum or platinum alloy hypodermic needle. For withdrawing fluids from flask and tube cultures. It should be 17 gauge and, for flasks, about 50 mm. in length. For tube cultures, it should be somewhat longer.

PACKING INSTRUMENTS

For this purpose metal boxes, if available, are excellent, but large Petri dishes (200 mm.) or flat covered tins (film tins do nicely) are quite serviceable. The instruments are laid in the containers, layer upon layer, each layer being separated by pads. This protects the instruments from injury and also from dust after the box is opened. The pads are about 3-mm. thick and may be made of absorbent cotton covered with black sateen or other lint-free material.

Bleeding instruments. Before packing, thread the needle with surgical silk of medium weight. Attach 150 to 175 mm. of ligature silk to each of two small curved hemostats. Protect the scalpels and artery scissors by wrapping the edges with absorbent cotton. (Do not use cork for this purpose because it will discolor the blade when sterilized.) Then arrange the instruments in a container (a 100 × 200 mm. moist chamber does very well), in the order they are to be used: the threaded needle and the silk scissors on the bottom; the fine scissors, curved forceps, serrefine and three curved hemostats next; the knives and hemostats for swabbing and holding the skin, on top.

Extract instruments. Place them on a pad in a 150 mm. Petri dish or small metal box.

Culture instruments (Fig. 8). Place a pad in the bottom of the metal box or Petri dish (25 × 200 mm.), place a row of knives on it, and a coverslip forceps beside them, cover with another pad. When a knife is needed, the edge of the covering pad is raised by means of an attached clamp, and one knife removed without uncovering the others. It is unwise to sterilize the cataract knives unnecessarily and, when cultures are being washed only, knives will not be needed. Therefore, the remaining forceps to be used for removing the small coverslips for washing should be packed in a separate dish or in individual test tubes.

Some dissecting needles and a knife blade for removing paraffin from slide cultures are sterilized in a Petri dish. The boxes are then covered and wrapped in paper ready for sterilization.

The work is greatly facilitated by having at least two separate sets of extract and culture instruments ready for use. Instruments of good quality should be obtained, and care taken to keep them in proper condition. Never allow organic matter, such as tissue, blood, etc., to dry on them. As soon as possible after use they should be rinsed with cold water, after disconnecting those which have removable joints. Brush thoroughly in warm water and soap, rinse in hot water, then in alcohol, and finally wipe with a clean towel. Be very sure the instruments are absolutely dry before packing them in the air-tight containers, otherwise they will rust.

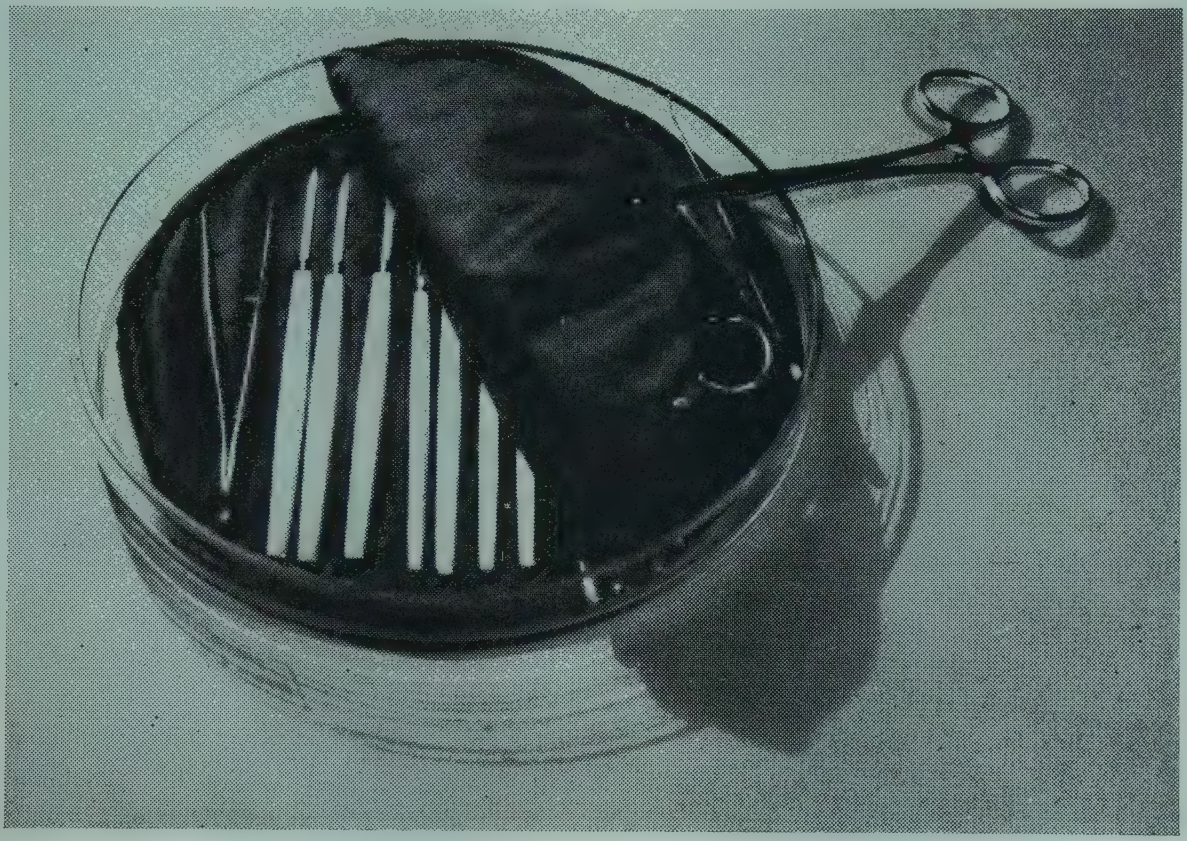


Fig. 8. Arrangement of culture instruments, packed for sterilization.

Every few days the cataract knives should be sharpened on a Charles Emerson razor strop. A few strokes on the brown side and then a few on the black will usually keep them in good condition. If this type is not available, a coarse (brown) and a fine (black) block strop serve very well. If necessary the knives may be sent to the factory occasionally for reconditioning. It must be remembered that cultures cannot be properly cut with a dull knife.

ACCESSORY EQUIPMENT

There are certain miscellaneous items which are necessary or desirable for a tissue culture laboratory, for example:

Board for culture table. A smooth level board, 7 × 16 × ¾ inches, which is placed on the center of the table with its narrow end flush with the front edge of the table. The board serves as a slightly raised platform on which to work. By allowing the hands to rest on the table at each side of the platform, it ensures more accurate control and reduces fatigue when delicate manipulations are to be performed.

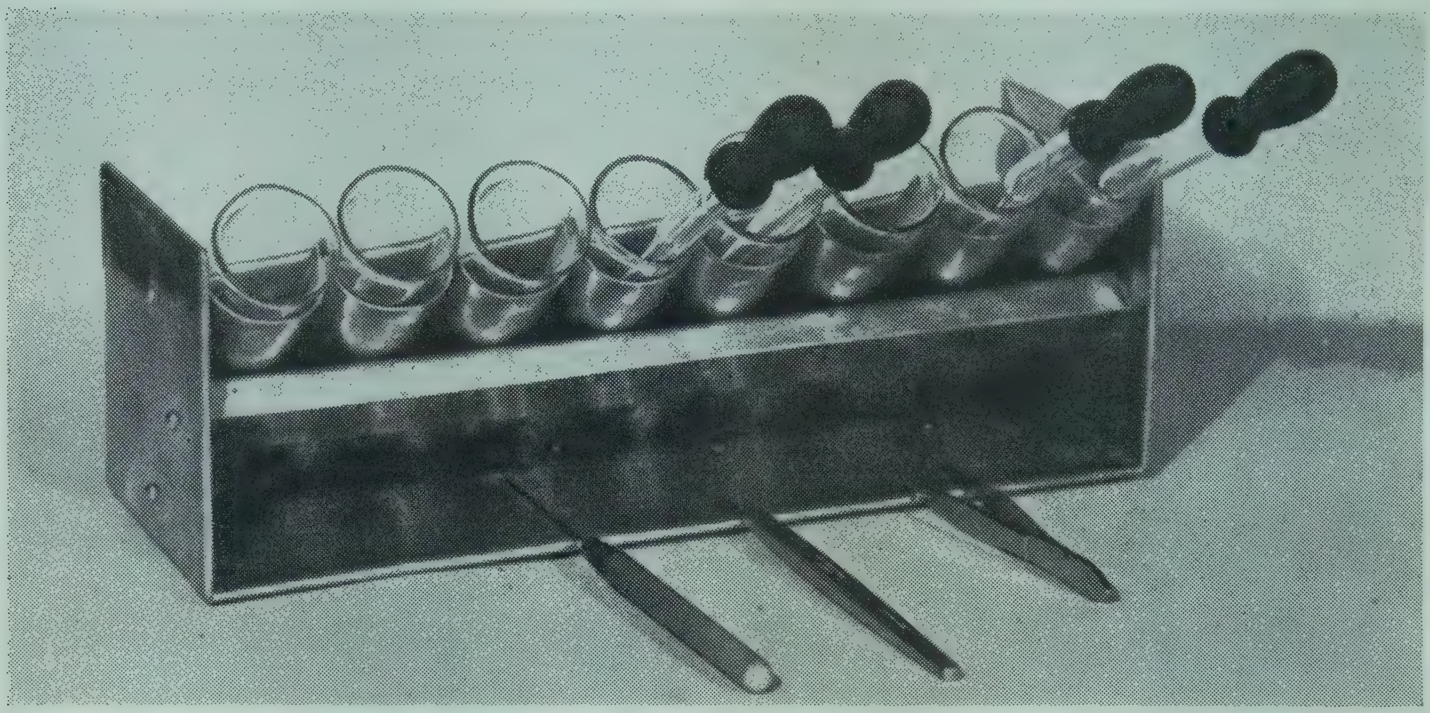


FIG. 9. Metal rack for holding pipettes in tubes and with shelf for instruments.

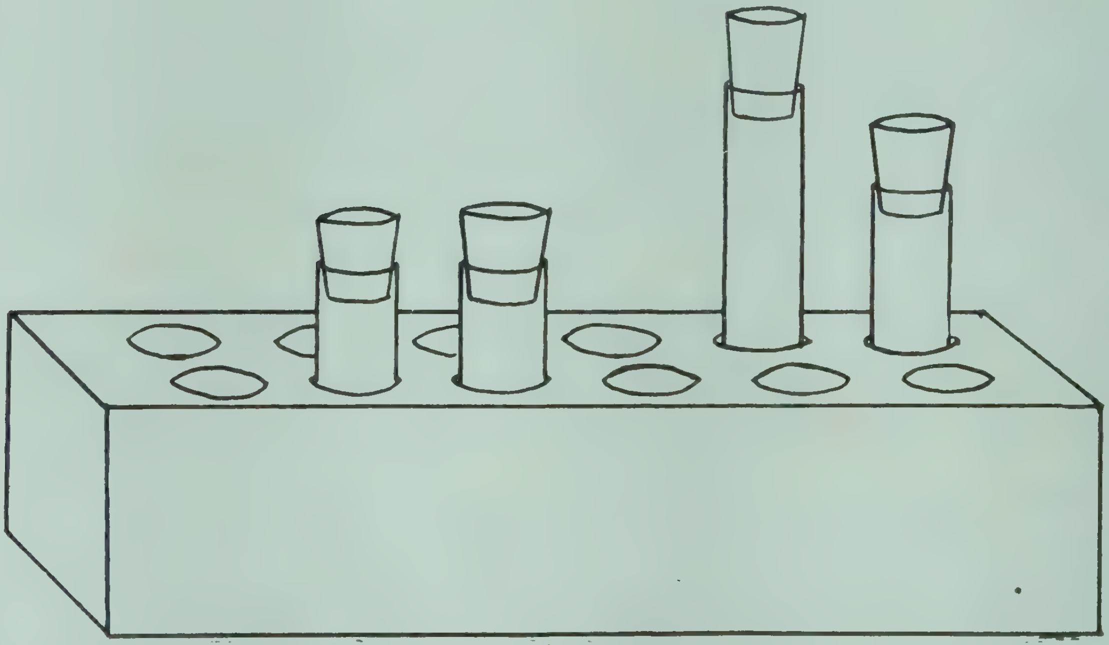


FIG. 10. Wooden block with perforations for tubes.

Bakelite square. A small square of black glass or bakelite about 3½ × 2½ × ½ inches on which cultures for transfers are cut.

Metal rack (Fig. 9). This rack, 9 × 3 × 3 inches, of aluminum or monel metal, similar to one devised by Carrel, is for holding pipettes and instruments while in use, at the same time protecting them from contamination by dust. Sterile tubes to hold the pipettes are placed in the grooves of the top compartment, which is slanted to prevent fluid from running into the nipples attached to the pipettes and to prevent dust falling into the tubes. Instruments are placed on the tray beneath. The rack is sterilized each time before using.

Wooden block (Fig. 10). In a block of wood 10 × 4 × 2 inches, two rows of six vertical holes 1 inch in diameter may be made to hold tubes of media for storage in the icebox and for use during culture making.

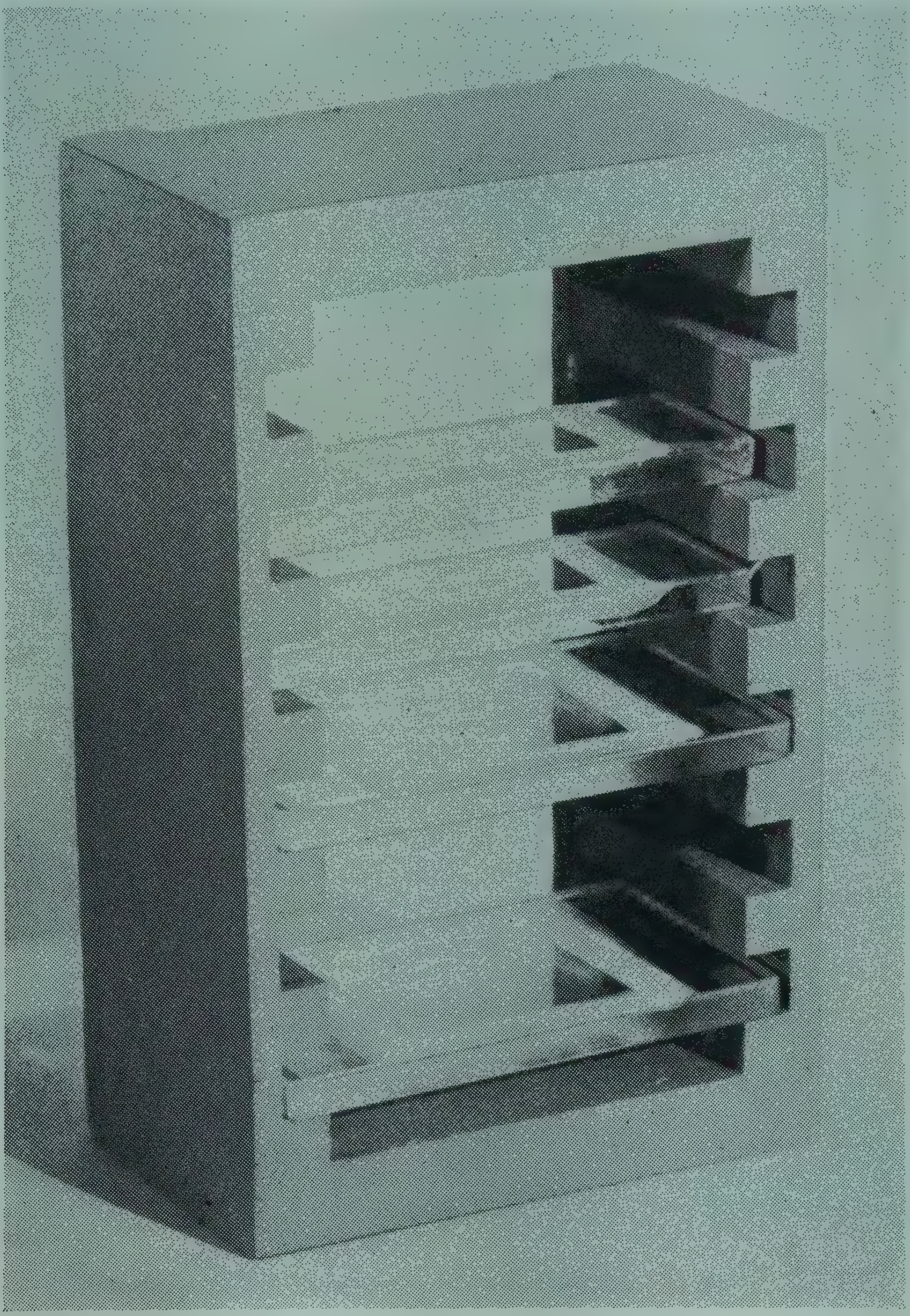


FIG. 11. Wooden rack for holding slide cultures.

Wooden block. Similar to the one above but with the perforations drilled at an angle so that when tubes must be left unstoppered during transfer of fluids, dust will not fall directly into the tube.

Incubator racks (Fig. 11). Incubator racks, 5½ × 3½ × 2½ inches, developed in this laboratory, are made of wood and hold 6 slide cultures either face up or in an inverted position, as desired. A narrow strip of wood at the back prevents the slides from slipping off the ledges but does not interfere with the circulation of air, which might alter the temperature. The racks economize space in the incubator and are useful for keeping a series together.

Chicken board. A board, 16 × 14 × 1 inches, has metal cleats at each corner to which the fowl is tied for bleeding. Two sand bags, 12 × 5 inches and about 5 lbs. in weight, are used with this to hold the wings in position.

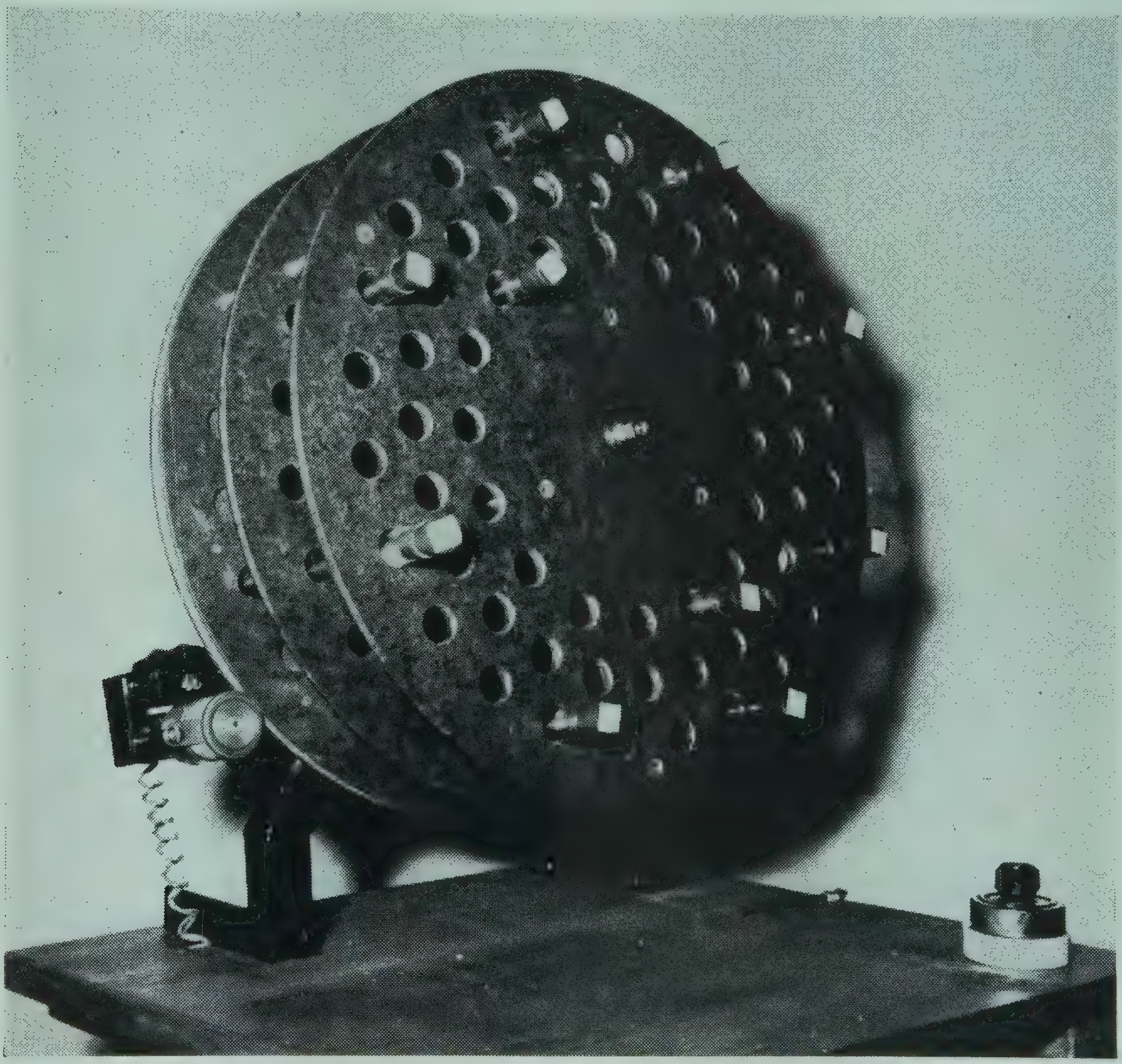


FIG. 12. Roller tube drum for holding and rotating tube cultures in incubator.

Roller tube drum (Fig. 12). Introduced by Gey (1933) to hold roller tube cultures. It may be made of metal or fiberboard and is designed to hold as many tubes as desired, usually from 60 to 100. The drum is placed in an incubator and rotated by a small motor 7 to 10 times per hour. The tubes are held in an almost vertical position with the holder set at an angle just sufficient (about 5 degrees) to prevent the supernatant fluid from touching the stoppers. The revolution of the drum exposes the tissues alternately to the fluid and gaseous phases of the medium. See Gey (1933), Gey and Gey (1936), Lewis (1935b), Coman and Stabler (1941) and White (1946) for further details on construction.

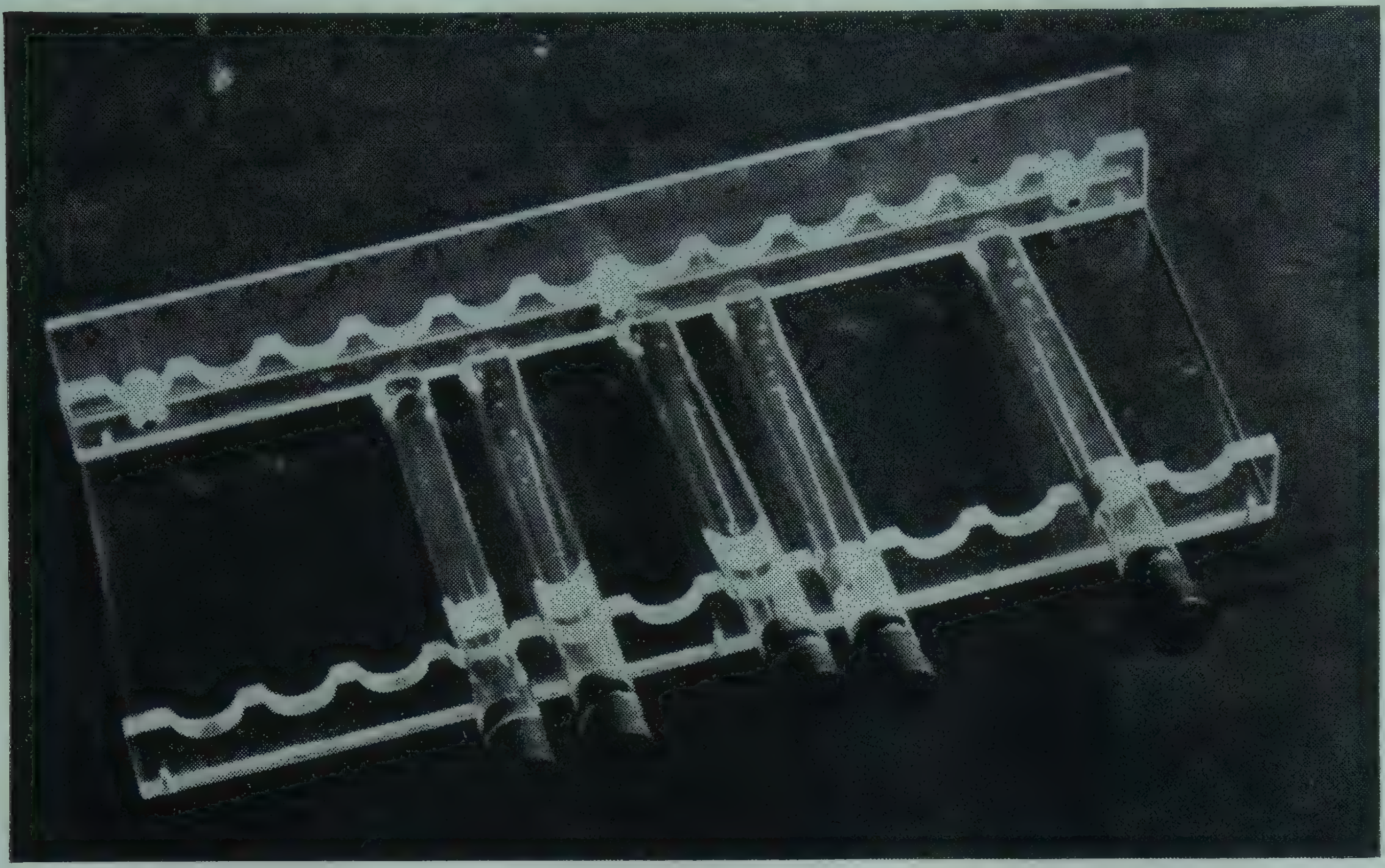


Fig. 13. Lucite rack for holding roller tubes during manipulation.

Rack for roller tube cultures (Fig. 13). In this laboratory we use a rack made of plastic to accommodate 12 tubes during preparation of tube cultures and change of medium. It may be made of wood if preferred. The grooves are arranged so that the tubes may be placed in a horizontal position during coagulation of the medium or at a slight angle when they contain fluid.

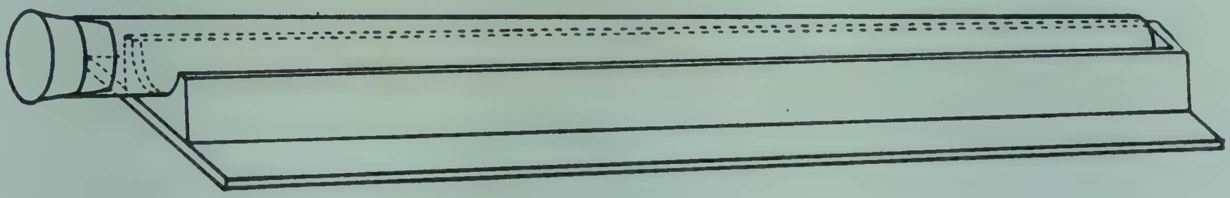


Fig. 14. Holder for roller tube during microscopic examination.

Microscope holder for roller tube (Fig. 14). Made of metal to hold the tube in position for microscopic examination. One end is slightly raised to prevent the fluid from touching the stopper.

Holder for Carrel flasks (Fig. 15). When stoppered, the flasks have a tendency to tip, allowing the fluid to run into the neck—a possible source of contamination. To prevent this, in our laboratory we use (after Earle) a block of wood, 8 × 2 × ½ inches, to which metal clips are attached in such a way that a flask may be slipped under each clip to hold it in position. The clips are spaced to accommodate either 3.5- or 5-cm. flasks.

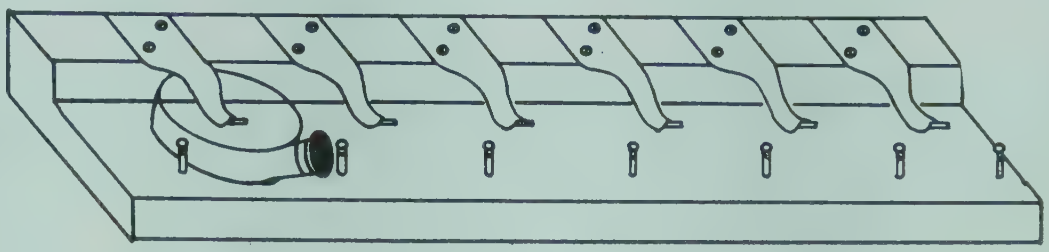


FIG. 15. Block for holding Carrel flasks in position during incubation (after Earle).

ACCESSORIES OF A LESS SPECIFIC NATURE

Gowns, masks, etc. Clean, washable cotton gowns should be worn at all times in the laboratory. During operative and culture work muslin masks over the nose and mouth and a covering for the hair should be worn.

Unbleached muslin. This material is used for making covers in which supplies to be sterilized by steam are wrapped. A loosely woven quality is best, because sterilization thickens the cloth. Cut and hem pieces of suitable size for the various materials to be wrapped, always allowing enough to make the cover of two thicknesses of muslin. A sufficient number of covers should be made to allow for laundering occasionally, because repeated steam sterilization stiffens them.

Black sateen. Excellent for use as table covers and as a sheet for bleeding operations. It is easily sterilized and lint-free. Other cotton material may be used, but the uniform use of black sateen is restful for the eyes; it prevents reflections; it is a good background for transparent tissues; and it prevents slipping of the glassware. At least four covers should be made—two for the culture table, one for the operating table of such a size that the table and its edges will be completely covered. The fourth, 1 to 1½ yards long, is used for covering the chicken when it is being bled. In the center of this sheet, 4 or 5 inches from one edge, cut and hem a slit about 4½ inches (100 mm.) long. This sheet is placed over the bird and the table on which it lies in such a way that everything is covered except that part of the animal which is to be operated on. Each cover is then folded flat and wrapped in a muslin cover for autoclaving.

Surgical gauze. Used chiefly for making sponges or swabs. It may be obtained by the bolt, fifty or a hundred yards. Cut pieces about 200-mm. square and fold in the cut edges to make a neat and convenient sponge about 75-mm. square. There should be no loose, ravelled edges. Wrap six to eight in a muslin cover for autoclaving.

Rubber nipples for pipettes. Heavy black rubber dropper nipples of about 2 cc. capacity are to be desired but have been very difficult to obtain recently. However if and when obtainable they, as well as all new rubber supplies, should be boiled with sodium bicarbonate to remove possible traces of acid, then scrubbed clean and thoroughly rinsed. Dry those not to be used at once and store them in the refrigerator. Place about a dozen clean nipples in each of two half-pint mason jars containing an inch or two of distilled water; wrap the jars in muslin covers to be autoclaved. If sterilized under pressure without water more than once or twice, they lose their elasticity. If black nipples are unavailable, those made of red rubber or natural latex must be used. These do not withstand repeated sterilizations well and it may be necessary to dispense with it. If the pipettes are sterilized with a plug of absorbent cotton inserted in the top, non-sterile nipples may be safely used.

Solid rubber stoppers. For roller tube cultures the stoppers should be of good quality rubber with a low sulfur content. These should be cleaned as described for nipples. When thoroughly dry about six to eight are placed in a test tube with the larger end toward the opening of the tube, which is covered with two layers of paper ready for autoclaving.

"No-air" red rubber stoppers. These are excellent for Carrel flasks. They have a solid rubber core with a protecting flange which prevents contamination of the rim of the neck of the flask. Size No. 1 fits the 3- or 5-cm. flask and size No. 2 may be used for roller tubes. After they are washed and dried the thin upstanding flap of rubber should be turned down over the solid central portion before they are sterilized. A convenient method of packing them for sterilization is to stand them, top side up, on a circle of glossy paper (weighing paper is good) in an open Petri dish, which is then wrapped in muslin for autoclaving.

Yellow vaseline. To be used for preliminary sealing of cultures and for protection of an exposed blood vessel during bleeding. The yellow variety should be used, since white vaseline is likely to contain acid used in bleaching it. Fill three or four small jars one-third full, then cork and tie two layers of paper over the cork for autoclaving. During culturing, the cork is replaced with a circle of filter paper with a small perforation in the center to accommodate a glass rod. The paper may adhere to the rod but its light weight will not interfere with its use in the temporary sealing of the cultures.

White blotting or filter paper. Strips (about 75 × 15 mm.) autoclaved in a Petri dish are used to absorb excess moisture after washing double coverslip cultures, for absorbing moisture in draining tube cultures or for removal of fluid medium from coverslip cultures.

Nail brushes and towels. These should be packaged and sterilized for operative work.

Microburners. Used for flaming opened flasks and tubes during culturing.

Paraffin is required to seal cultures and should be in an enamel cup. Parawax is of the right consistency, or a low melting point embedding paraffin (about 45 degrees) may be used. In that case it is well to add about 5% yellow vaseline to prevent cracking of the seal.

Camel's hair brushes are needed for application of the paraffin.

Electric hot plate. Used to melt the paraffin.

CHAPTER IV

STERILIZING PROCEDURES

Cleanliness, care and common sense are prerequisites for success in tissue culture and strict sterile precautions are necessary throughout the entire routine. The tissues to be cultivated and the biological media must be obtained in an aseptic manner and everything else to be used, viz., glassware, instruments and physiological solutions, etc., sterilized in one way or another. For these procedures some knowledge of bacteriological technique is helpful; but this can be acquired, and the special requirements will be noted as we come to them.

Common sources of contamination to be guarded against are: currents of dust-laden air, fingers, breath, insufficient sterilization of instruments and of glassware, etc.

The *culture room* must be kept clean and free from currents of air which may circulate bacteria-laden dust. In laboratories designed expressly for tissue culture, equipment for dust control and a supply of clean filtered air is usually installed and is very helpful. But where it is necessary to use regular laboratory space, some workers use a spray of some kind, possibly germicidal, to precipitate the dust in the air a short time before culture work is to be done. In our laboratories, we rely on ordinary cleanliness (which should not be underrated)* with frequent washing of the floors and careful dusting of the furniture, ledges, etc., with a damp cloth. Many workers use a protective cage, which may be of glass or plastic, over the culture table. When the culturing is done in a small separate room, we have found this unnecessary. If, however, several people must work in the same room, such protection is warranted.

No unessential activity should be permitted in the culture room while culturing is imminent or in progress since every movement in the room disturbs the air and enhances the danger of dust contamination. This includes such possible preliminaries as operations on animals or obtaining blood or tissues from them. Fur and feathers do fly.

The tissue culture worker should wear a clean, washable dress or coat with short sleeves and a covering for the hair. The hands must be kept scrupulously clean. Needless to say, smoking must not be permitted in the culture room. Once the sterile work is in progress, the tissue culture room and the workers should be undisturbed. Danger of breath contamination is lessened by wearing a muslin mask over the nose and mouth and above all by talking as little as possible. The operator should sit straight on a low stool so that he will not find it necessary to lean over the table. This sounds unimportant, but experience has shown it to be quite the contrary. Constant watchfulness is necessary, and the operator must be on the lookout for any break in the sterile technique. Whenever there is any doubt about the sterility of an article, it should be discarded.

---

* Florence Nightingale referred to Pasteur as "That gentleman in Paris who claims that disease is due to germs." "I know better," said Miss Nightingale. "It is due to plain dirt."

The preparation of equipment and media is of no less importance than the actual making of the cultures themselves. Each step is simple, but time and care must be given to it. Foresight is an essential virtue in this connection. The technical procedure should always be well planned, and all necessary materials on hand and within easy reach before beginning any operation.

Since all materials used in the preparation of the cultures must be sterile, it adds greatly to the efficiency of the work if the required materials are stored in a sterile condition to be ready when needed. For this purpose everything is wrapped before being sterilized and, when removed from the sterilizer, should be stored in closed cupboards to prevent the entrance of dust. Even so, as a precautionary measure, any article unused for two or three weeks should be resterilized before using.

The packaged *glassware* and *instruments* (see Chapter III) are sterilized by *dry heat*. Except in an emergency, boiling of instruments is not recommended as water remaining on them is a likely source of contamination and possible injury to some materials. All sterilized materials must be allowed to cool before being used.

The glassware, together with the instruments, is sterilized by heating for 1 hour at 150°C. It is important that this temperature be maintained as closely as possible. The temperature should not drop below this point to ensure the killing of spores, and if it should rise much higher, alkali will be liberated from the glass. The knives also must be guarded from overheating, or their temper will be lost.

Articles which would be spoiled by intense dry heat are sterilized by *steam* for 30 minutes in an *autoclave* at 15 lbs. pressure. These include all cloth materials such as table covers, gowns, towels, gauze swabs, etc. Rubber stoppers, jars of rubber nipples and of vaseline, Ringer solution and the cannula (in oil) should also be autoclaved. The danger of breakage of glass containers in the autoclave is enhanced if glass touches glass. Therefore, unwrapped articles such as flasks of Ringer solution, etc., should be separated from each other by a towel or a cloth wrapped package.

When the steam sterilization is completed, turn off the heat and allow the pressure to drop gradually. Do not open the steam outlet until the pressure is almost down to normal. Otherwise the corks will probably blow out of any stoppered containers. Open the door slightly and leave the cloth materials in the sterilizer until cool and dry. If removed too soon or if the door is entirely closed they will be damp, and moisture provides a means of entrance for bacteria. The various packages should then be stored in the closed cupboards provided for that purpose.

If an *Arnold sterilizer* must be substituted for an autoclave, the time of sterilization should be increased to 1 hour, since this type of sterilizer produces free steam only and no pressure. Solutions or any material actually used in the culture medium should be sterilized for ½ hour on three successive days. This is in order to give the spores time to develop for, as spores, they may not be killed by Arnold sterilization.

Solutions which would be chemically altered by heat sterilization, e.g., Tyrode solution, may be sterilized by *filtration.* This will be discussed in the following chapter (V).

The use of chemicals and antibiotics for sterilizing infected tissues is described in Chapter IX.

CHAPTER V

PHYSIOLOGICAL SOLUTIONS

Some one type of physiological salt solution is used in the preparation of practically all cultures and is an essential constituent of most culture media. Its general use both as a diluent for biological media and in the irrigation of tissues makes its preparation, chemical composition, hydrogen ion concentration, sterilization and storage of paramount importance. There are numerous serviceable physiological salt solutions but the inorganic salt content and osmotic pressure of whichever one is used should duplicate as nearly as possible those of normal serum. Two chief considerations for the environmental fluids of cells are maintenance of a pH which is on the alkaline side of neutrality, and proper osmotic conditions. Cells in tissue culture can withstand some degree of hypotonicity, but proportionally a much smaller degree of hypertonicity.

The formula for Tyrode solution is the basis of most of the physiological salt solutions used. Various investigators have made slight variations in its composition to meet their specific needs, but the original Tyrode solution is widely used and the simplified method of preparation used in our laboratory will be discussed first. Variants will be taken up later and the formulae for other frequently used solutions will be found at the end of the chapter.

FORMULA: TYRODE FOR WARM-BLOODED ANIMALS

$NaCl$	8.00 g.
KCl	0.20 g.
$CaCl_2$ anhydrous	0.20 g.
$MgCl_2$	0.10 g.
NaH_2PO_4	0.05 g.
$NaHCO_3$	1.00 g. (we use 0.7 g.)
Glucose	1.00 g.
Redistilled water	1000.00 cc.

pH 7.4 for mammalian or 7.8 for chicken material

MATERIALS REQUIRED

Redistilled water
Salts listed above
1% solution of phenol red

1000 cc. graduated cylinder
1000 cc. beaker
250 cc. Erlenmeyer flask
2 liter Erlenmeyer flask
Glass rod for stirring
Rubber stoppers to fit flasks
Cork stoppers (covered with cellophane) to fit flasks
Analytical balance

The water should be double or triple distilled, the last distillation, at least, being through a Pyrex-glass still into a Pyrex flask. Distillation is necessary to eliminate the mineral content, and is also important for the removal of any endotoxins* which may have been produced in the water by Gram-negative organisms. If not used immediately, the flask of water should be tightly closed with a rubber stopper.

The salts for any solution for tissue culture should be the purest obtainable with emphasis on their freedom from traces of heavy metals, the presence of which is inconsistent with biological requirements. Sometimes it is necessary to try out several lots before satisfactory ones are found, noting that jars of the same brand may differ from time to time.

One of the important factors in the maintenance of suitable environmental conditions for cells *in vitro,* is the provision of a favorable hydrogen ion concentration in the medium. In general, for chick tissues, the optimum is pH 7.8, for mammalian tissues it is 7.4 to 7.6. Phenol red (P. R.) is a very useful pH indicator for tissue cultures. It has a range from 7.0 to 8.2 assuming a yellow color at 7.0 or less, which gradually changes to pink at 7.6 to 7.8 and to purplish red at 8.2 or higher. It is not altered by autoclaving and is practically nontoxic. Therefore, a quantity of the indicator just sufficient to indicate the pH may be added to any culture medium without affecting the cells. By the addition of 4 drops of a 1% solution to a liter of stock physiological solution the pH of the solution can be determined at a glance and any subsequent changes on storage noted immediately and corrected.

Many beginners experience difficulty in obtaining a satisfactory Tyrode solution. The chief sources of trouble are the formation of a precipitate (calcium carbonate) or of excessive alkalinity. However, by following the directions carefully, both of these difficulties may be avoided. It will be noted in the following paragraph that the total initial amount of water is 1100 cc. instead of the standard 1000 cc. As already mentioned, nearly all cells will prosper in a slightly hypotonic environment and the extra water is to make due allowance for evaporation during autoclaving, for manipulation of the tissues during exposure to air, and for any possible loss of water from the finished culture medium by condensation.

---

* Personal communication from Dr. Jean Broadhurst, Professor Emeritus of Bacteriology of Columbia University.

Measure 950 cc. of redistilled water into a large beaker and add 4 drops of 1% solution of phenol red. Weigh and add each of the first five salts to the water separately and in the order listed, stirring the solution until each is completely dissolved before adding the next. Now add the glucose and pour the mixture into a 2-liter volumetric flask. The sodium bicarbonate is prepared separately by measuring 150 cc. of redistilled water into a 250 cc. flask and adding 0.7 g. of sodium bicarbonate. There are now two flasks, one of which contains 950 cc. plus salts, and the other 150 cc. plus the bicarbonate. Place the flasks on a steady table and mark the level of the fluid with a diamond pencil. Stopper the two flasks of solutions with corks covered with cellophane and tie a double layer of paper over the cork and the neck of the flask. As soon as possible autoclave at 15 lbs. pressure for ½ hour since the presence of glucose in the solution encourages growth of bacteria. The cellophane is inert and condensation of steam on it which may later drop into the solution is harmless. Rubber stoppers to fit the flasks when finally stored should be wrapped and sterilized at the same time. Rubber stoppers are better than cork for final storage, but they should not be used during autoclaving because of their tendency to be drawn into the flasks.

If the flasks have been properly stoppered and the autoclaving controlled to prevent sudden changes of pressure, particularly when the pressure is being released at the termination of the sterilization, the volumes, when the flasks are cooled, should closely approximate those before autoclaving. The volume of the liquid in the flasks must be noted before and after autoclaving for assurance that no appreciable loss of water has occurred. If an appreciable loss of water has taken place the solution should be discarded. A partial correction of the condition may be made by adding sterile distilled water to compensate for the evaporation. The solution containing the phenol red will be yellow because of the acid phosphate present.

Let both solutions remain in the autoclave until they are cold. Then add enough of the sodium bicarbonate solution to the larger flask to obtain the desired pH as demonstrated by the color change from yellow to pink as the solution becomes alkaline. If mixed while still warm a precipitate will form. If the entire quantity of the sodium bicarbonate solution is not required to obtain the desired pH, no ill effects to the tissues will ensue by adding less than the full amount of 150 cc. because 100 cc. of extra water has been provided for this eventuality. If the Tyrode is to be used for tissues from various organisms, e.g., mammals and chick, it is convenient to add enough $NaHCO_3$ to make the pH of the solution 7.4 only for mammalian tissues (probably about 100 cc.); when the solution is needed for chick material the addition of a few drops of $NaHCO_3$ will be sufficient to bring it to 7.8.

When the two solutions have been mixed and the proper pH obtained, stopper the flask with a tightly fitting rubber stopper, already sterilized, replace the sterile paper cap for the protection of the lip of the flask and store at from 0 to 5° C. When cultures are to be made, a small amount is transferred to a small flask or tube, to obviate the possibility of contamination of the stock supply.

When $NaHCO_3$ is present in a solution, the pH is controlled by the $NaHCO_3$ present. On exposure to air, some CO_2 is lost and the solution may become unduly alkaline and thus harmful to the tissues. For this reason a solution without or with very little bicarbonate is recommended for washing the tissues while exposed to air. Tyrode without $NaHCO_3$ (Carrel's glucosol), Ringer's solution or Hank's with very little bicarbonate may be used for this purpose. Glucosol, Ringer or any solution without $NaHCO_3$ may be sterilized by autoclaving. It has been found that $NaHCO_3$ is important in the glycolytic activity of cells and therefore is an essential constitutent of any medium. Solutions without $NaHCO_3$ are protective only and not recommended for any considerable proportion of the culture medium.

There are various other methods of preparing and sterilizing physiological salt solutions. For many years the standard method of making Tyrode solution consisted of adding all the ingredients to 1000 cc. of water and sterilizing it by filtration through a Berkefeld filter. This method is still used in some laboratories. The main advantage of this is that the osmotic pressure of the solution is unaltered. Our experience has been that the disadvantages outweigh this advantage. The filters are expensive and their preparation, cleaning and drying, time consuming. This task must be carefully and thoroughly done or the solution will be useless. Immediately after use, before the salts can dry in it, clean the filter by drawing several liters of water through it in the opposite direction to which the Tyrode was put through. Dry over night in a drying oven before sterilizing. Inadequate washing of the filter frequently results in faulty Tyrode solution and consequently poor cultures. Since tap water usually contains some discoloration from the pipes, all of which is picked up by the filter, it is safer as a routine to use only distilled water for washing the filter. When a candle becomes discolored, it must be discarded and a fresh one prepared from a reserve supply. Both

Berkefeld and Seitz filters tend toward alkalinity which is increased by the loss of CO_2 occurring with suction filtration. The filtered solution therefore becomes unduly alkaline. If Seitz filters are used, the discs should be soaked in $N/10$ HCl, rinsed and dried before use. The newer porcelain Selas filter and the Bush positive pressure filter of sintered glass are preferable, as little alteration of pH of the filtrate occurs.

These two filters are very useful for any materials which would be altered by heat sterilization, and are routinely used in some laboratories for sterilization of all physiological salt solutions. Another frequently used method for the preparation of stock salt solutions consists of making a solution of each salt separately and combining the required amounts when needed. The Na, K, Ca and Mg salts may be stored indefinitely in solution, but the acid phosphate and the bicarbonate are less stable and the glucose is subject to bacterial contamination. Therefore, the last three ingredients should not be stored for long periods.

If for any reason the presence of even a very small amount of phenol red is contraindicated, the indicator is omitted when making the solution. In this case its hydrogen ion concentration must be ascertained after sterilization. This may be done by testing a small amount of the solution in a potentiometer, if one is available. However, the colorimetric method is simple and usually sufficient for this purpose. Into a clean narrow tube pour 5 cc. of Tyrode and add 0.5 cc. of 0.04% phenol red. By comparing the color with a set of standard buffers, the pH is quickly obtained. If small amounts of solutions are to be tested, the drop method of pH determination may be used. Rinse a porcelain test plate with distilled water and when dry place a minute drop of each of several indicators (0.02%) which would be expected to cover the pH range, in separate drops at intervals on the plate. Brom cresol purple, pH 5.2 to 6.8, phenol red, pH 6.8 to 8.2, and cresol red, pH 7.2 to 8.8 would be suitable. Connect a drop of the solution to be tested with a drop of indicator and compare the resulting color with the color chart obtainable for this purpose. Instead of the color chart, the test may be made by comparison with drops of buffer solutions of known pH value colored with the pH indicators. The readings must be taken immediately since, with exposure to air for more than a few seconds, any bicarbonate containing solution will lose CO_2 and the result be invalidated.

When, at any time, the pH of the Tyrode is unsatisfactory, the question of adjusting it arises. If the cause of the change is unknown, it is wiser to discard the solution whenever possible and prepare a fresh lot. In cases of emergency, however, adjustments can be made as follows: if the solution is too alkaline—and this is the more likely occurrence—add a few drops of $N/10$ HCl or NaH_2PO_4, sterilized by boiling, and mix well. Or bubble CO_2 through it. To do this, sterilize a piece of glass tubing and put cotton in one end to act as a filter; attach by rubber tubing to a CO_2 tank. If a tank is not available, a small amount of solution may be altered by blowing one's expired breath through the cotton. Obviously, this is only a makeshift that may endanger sterility. If the Tyrode solution is slightly too acid, add more of the $NaHCO_3$ solution or a few drops of $N/10$ NaOH (sterilized by boiling). If the change on the acid side is marked, however, contamination should be suspected and bacteriological cultures made if a fresh supply cannot be obtained. Refiltering infected solutions is not advisable, since the presence of bacteria results in chemical changes in the solution.

Bacteriological tests are routine for tissue culture media in many laboratories. Whether they are necessary or not will depend on individual results. In any case, it is well to have on hand for such purposes a supply of agar slants and glucose broth in the refrigerator. These tubes of media are very easily prepared, since nutrient agar and broth powders of many varieties are on the market. They are supplied already filtered and with the pH adjusted, so that it is merely necessary to add the stated quantity of distilled water and to sterilize. Fill test tubes (150 × 15 mm.) to about one-third, plug with nonabsorbent cotton and autoclave. The tubes of agar are filled about one-sixth full, plugged and autoclaved. The tubes should be allowed to stand at an angle until cool. The agar will then be firm, with an oblique surface about 90-100 mm. long, presenting a wide area for growth. At present in this laboratory we keep on hand a supply of Brewer's thioglycolate (Difco) medium in which both aerobes and anaerobes grow well. The aerobic organisms, such as molds, will multiply in the upper third of the medium and the anaerobes toward the bottom of the tube.

Since the object in making bacteriological cultures of tissue culture media is to determine whether any organisms whatsoever are present, at least 0.5 cc. of the suspected material should be used for inoculations instead of the usual loopful. The cotton plug should be removed from the tubes with a slight twist and held, while making the inoculation, between two fingers in such a way that the hand does not touch the part to be replaced in the tube. The mouth of the tube should be flamed, and a fresh sterile pipette used for each substance to be tested. For anaerobes, after the tube of broth has been inoculated, melted sterile paraffin or agar should be introduced and allowed to harden to form an air tight plug.

Incubation of from 48 to 72 hours should be allowed before deciding that a suspected material is sterile. If bacteria are present, the colonies will readily be apparent on the agar slants and the broth will be cloudy. If there is any doubt compare with an uninoculated tube.

The following formulae of physiological salt solutions for warm- and cold-blooded animals are frequently used.

PHYSIOLOGICAL SALT SOLUTIONS FOR WARM-BLOODED ANIMALS
FOWLS AND MAMMALS

	Grams per 1000 cc. H_2O										
Solution	P. R.	NaCl	KCl	$CaCl_2$ Anhydrous	$MgCl_2$ $6H_2O$	$MgSO_4$ $7H_2O$	NaH_2PO_4 H_2O	Na_2HPO_4 $2H_2O$	KH_2PO_4	Glucose	$NaHCO_3$
Tyrode		8.00	0.20	0.20	0.10		0.05			1.00	1.00
Gey (1936)		8.00	0.30	0.275	0.210			0.150	0.025	2.00	0.250
Earle (1943)*	0.05	6.80	0.40	0.20		0.10	0.125			1.00	2.20
Hanks (1949)**	0.02	8.00	0.40	0.20***		0.20		0.06	0.06	1.00	0.35
Glucosol (Carrel)†		8.00	0.20	0.20	0.10		0.05			1.00	
Simms X7††	0.05	8.00	0.20	0.147	0.20			0.21		1.00	1.00
Locke		9.00	0.42	0.24							0.20
Ringer		9.00	0.42	0.25							
Locke-Lewis†††	85.0 cc. Locke solution, 15 cc. Chicken bouillon, 0.5 Dextros										

* cf. Earle, 1934.
** cf. Hanks, 1948.
*** Now using 0.14 to provide 5 mg. per cent Ca.
† Parker, R. C., 1938.
†† Simms and Sanders, 1942.
††† Lewis and Lewis, 1911b.

PHYSIOLOGICAL SALT SOLUTIONS FOR COLD-BLOODED ANIMALS

		Grams per 1000 cc. H_2O							
Solution	For	NaCl	KCl	$CaCl_2$	$MgCl_2$	$MgSO_4$	$NaHCO_3$	NaH_2PO_4	Glucose
Holtfreter (1931)	Amphibians Fresh water fish	3.50	0.05	0.10			0.20*		
Ringer, frog	Amphibians	6.50	0.14	0.12		0.20			
Sea water**	Squid and clams***	27.27	0.76	1.22	2.36	3.44	0.21		
Locke†	Insects	9.00	0.42	0.25			0.2		2.5
Carlson (1946)	Grasshoppers	7.00	0.20	0.02	0.10		0.05	0.20	0.80

(In general, adjust solutions if necessary to pH 7.2 to 7.4.)

* Prepare the $NaHCO_3$ and the salt solutions and sterilize separately. Mix when cold. Adjust amount of $NaHCO_3$ until pH 7.8.

** C. G. Rogers, Textbook Comparative Physiology, 1927, New York.

*** G. Cameron, unpublished.

† Lewis and Robertson (1916) also 2.0% peptone; for flat worms: M. R. Murray, 1928.

CHAPTER VI

PLASMA AND SERUM

Generally speaking, cells in tissue culture require a solid support for continued growth and activity. This may consist of the coverslip on which the tissue is placed or on a variety of solid structures introduced into the culture medium, for example, spider webs, cotton fibers, ravellings from silk or glass cloth, or cellophane. Much more efficient for prolonged growth are materials that form a soft coagulum, for example, agar, gelatine, fibrinogen or, best of all, blood plasma. The plasma is obtained by centrifugation of whole blood before coagulation takes place. The tissue is then placed in a small quantity of the plasma and coagulation encouraged by the addition of a small amount of tissue extract or thrombin.

Blood may be obtained from mammals and fowls or from many cold-blooded organisms when desired. Fowl plasma, although not the sought after perfect medium, has been found so far to be the most generally satisfactory and, in sufficient amount to form a coagulum, suitable for cells from any species. From a starved fowl, large amounts of clear, amber colored plasma may be obtained without the use of anticoagulants, and may be stored in paraffined tubes in the refrigerator for long periods to be ready when needed. In contrast to mammalian plasma which tends to become increasingly opaque with the age of the culture, the clot formed by chicken plasma remains optically clear, permitting unrestricted microscopic observation of the cells growing in it. An added advantage over mammalian plasma is a greater resistance of the fowl plasma coagulum to various lytic elements elaborated by some cells as growth progresses. Fowl plasma may be frozen or, as shown by Hetherington, lyophilized without detriment. Since fowl plasma has proven so valuable a base for culture media, lyophilized fowl plasma is now being prepared commercially under the auspices of the Tissue Culture Association. The blood is taken from young Plymouth Rock cockerels, a small amount of heparin added and the plasma pooled, lyophilized and sealed in small vials. The replacement of the water provides plasma equal in quality to that obtained from freshly drawn blood. This is a great convenience but, since many workers will still wish to prepare their own supply in the laboratory, and without heparin, the methods for obtaining it are described.

FOWL BLEEDING

The blood is obtained from the wing, heart, or carotid artery. The wing and heart methods are useful when small amounts only are required and have the advantage that they may be repeated many times on the same bird. The disadvantages are the possibility of contamination from the skin and the danger of coagulation from possible contact with tissue juices. The possibility of contamination may be overcome by incising the skin before inserting the needle; the danger of coagulation may be overcome by the use of heparin as an anticoagulant. With the carotid artery method, as much as 100 to 125 cc. of blood may be obtained without fear either of coagulation, or injury to the bird. Its only disadvantage is that the artery is destroyed, and therefore the bird can be bled only twice by this method.

Tubes to be used as containers for the blood and plasma are lined with paraffin to help prevent clotting. These are prepared beforehand in the following manner and stored in the refrigerator.

Materials Required for Making Paraffin Tubes

Granite pan (6 × 6 inches) with cover
Paraffin (melting point 56-62)
Tubes (75 × 18 mm.)
Corks to fit the tubes
Long curved forceps (sponge forceps)
Towels
Heavy paper

Into the granite pan with tightly fitting cover put 1½ or 2 lbs. of refined, filtered paraffin. Heat it just enough to melt, then gently drop about three dozen tubes into it. The pan must be tightly covered. It is advisable to tie paper over the cover for autoclaving because, if any steam should leak in, the paraffin will contain water and will not stick to the glass. Stoppers to fit the tubes (best quality cork obtainable) should be arranged so that when removed for stoppering the paraffined tubes, the fingers will not touch that portion of the cork entering the tube. They may be placed in long test tubes, top ends toward the mouth of the tube, or placed upright on a sheet of weighing paper in a flat tin (we use discarded film containers). The tin is wrapped in muslin taking care not to disturb the upright position of the corks. Metal trays perforated to fit the stoppers, as described by Parker (1938), are convenient too. The towels, folded flat and smooth, and the long forceps are also wrapped in muslin. A piece of rubber tubing may be slipped over the points of the forceps to prevent tubes from slipping.

Autoclave the packages (the pan containing the tubes and paraffin, the packages of corks, forceps and towels) for 30 minutes at 15 lbs. pressure. Spread a sheet of heavy paper on the table and cover with a sterile towel, being careful while doing so to touch only the corners of the towel.

While the paraffin is still boiling hot, with the long forceps remove each tube separately, open end down, and invert on the sterile towel to drain. After a few minutes when they are all cool, a slight twist as they are lifted will leave the excess paraffin on the towel, and ensure an even coat on the lip of the tube. Flame and cork each one as it is removed from the towel. Wrap the tubes in a fresh sterile towel and store in the refrigerator until needed. The paraffin in the covered granite pan may be set aside and used repeatedly.

BLEEDING FROM THE CAROTID ARTERY

A healthy young bird, not over a year old and preferably a rooster, should be selected. It should be young because growth-inhibiting substances have been found to exist in the blood in direct ratio to increasing age (Carrel and Ebeling, 1921). The male bird is to be preferred because it has more pronounced blood pressure and, incidentally, the large comb is convenient for holding the bird without pressure on the trachea during the operation. In order that no unassimilated food be in the blood stream to make the plasma cloudy, the chicken should have no food, but plenty of water, for 36 hours before the bleeding.

MATERIALS REQUIRED

Animal board
- Sand bags
- Strips of muslin bandage for tying
- Muslin cover for bird on board
- Ether and ether cone

Scrub-up table
- Sterile brushes
- Soap
- Sterile towels
- Sterile gown
- Alcohol (70%)

Operating table (sterile)
- Table cover
- Sheet (to cover bird)
- Sponges

Operating table (sterile)
- Beakers 2 (about 150 cc.)
- Warmed Ringer solution
- Cannula in oil
- Alcohol (70%)
- Box of carotid bleeding instruments

Paraffined tubes in ice

For withdrawing plasma
- Pipettes (sterile)
- Rubber nipples (sterile)
- Tubes to hold pipettes (sterile)
- Wooden block

Prepare a bowl of finely chopped ice and place about a dozen paraffined tubes in it. Loosen the corks slightly so that adherent paraffin will not interfere with the quick removal of the cork when a tube is needed. Place the bowl of tubes in the refrigerator until needed. Also, put the metal carrier tubes of the centrifuge in the refrigerator because, if they are thoroughly chilled, it will be unnecessary to pack the chicken blood in ice during centrifugation. Mammalian blood, if heparin is not used, must be surrounded by finely chopped ice at all times, including centrifugation.

An ordinary glass tumbler with absorbent cotton in the bottom may be used for giving ether, or better, a cone can be made from a quadrant of a 14-inch circle of cardboard formed into a cornucopia and covered with several layers of surgical gauze.

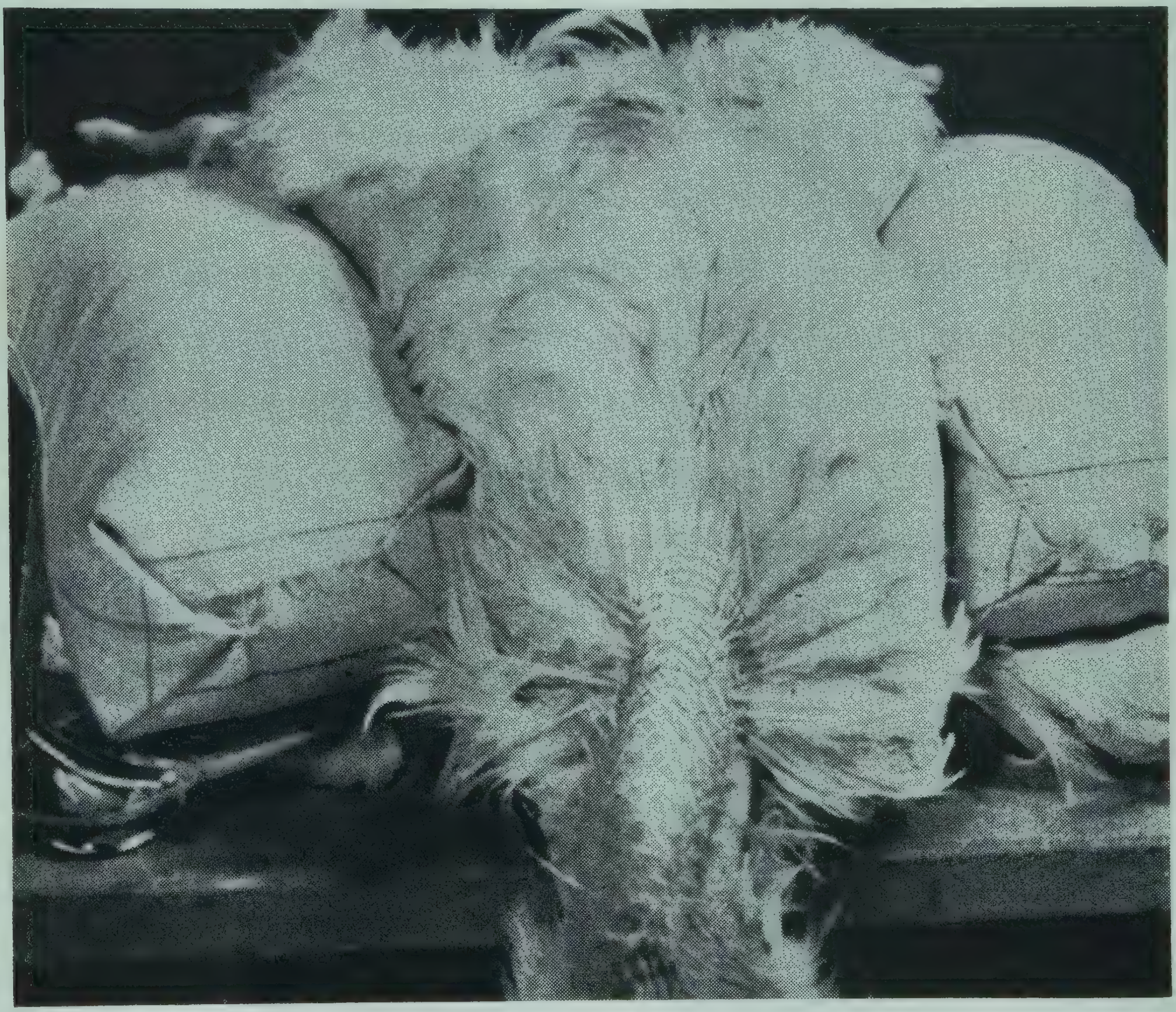


FIG. 16. Fowl on board in position for bleeding from carotid artery.

Place the bird gently on its back on the board (endeavor not to frighten it) so that the neck extends beyond the board. Tie the wings and the feet securely to the hooks at the four corners of the board. Put a sandbag on each wing and push them firmly against the body. Then wrap the board, fowl and sandbags in a large heavy towel or piece of unbleached muslin (about 1¼ yards long and ½ yard wide) and pin firmly but with consideration for respiration (Fig. 16). In this way movements of the bird out of position and scattering of feathers in the operating room will be prevented. Anesthetize the bird slightly and pluck the exposed upper side of the neck which is to be operated upon. (This should be done before the bird is brought into the operating room in order to obviate flying feathers.) Place the front edge of the board flush with the edge of the operating table so that the head and neck of the chicken will hang over the side of the table, directly in front of the operator.

The operator scrubs his hands thoroughly, rinses them in alcohol, and puts on a sterile gown. Gloves are not necessary and, at best, are somewhat clumsy for this delicate operation. The fingers need never touch the open ends of the cannula through which the blood will flow.

As the needed sterile supplies are removed from their packages they are placed on a table to the right of the operator. He sits on a low stool in front of the bird; the anesthetist is to his left. The assistant should then remove the bowl of paraffined tubes from the refrigerator and place them within easy reach. Using a sponge on a hemostat, the operator washes the plucked neck of the fowl with alcohol, and lays the operating sheet over the bird and the table in such a way that only the neck of the bird is visible through the slit. The neck should now be washed again with a fresh sponge in alcohol.

The comb of the rooster makes a convenient hold for the anesthetist, and its color is a helpful indication of the amount of ether that is being administered. The comb should always remain a bright red. Very little ether is needed and the chicken passes out quickly if given too much. It is a sign of sufficient anesthesia when a bird begins to breathe audibly and when there is no response to a touch on the cornea. No ether whatever should be administered after the flow of the blood through the cannula is established. The chicken almost always remains quiet; and tissues grow better in blood in which no trace of ether is discernible.

When the bird is quiet, make a ventral, longitudinal incision of about 100 mm. along the middle of the neck with a scalpel and clamp the edges of the skin to the edges of the slit in the cloth cover. Use a fresh knife for cutting through the subcutaneous tissues; then, with a small, blunt, curved hemostat, dissect out the right and left common carotid arteries which lie close together in the central, ventral groove of the cervical vertebrae. Select one and exposing its length for from 6 to 8 cm., remove any connective tissue clinging to it, and swab with warm Ringer solution. Ligate the distal end, and gently clamp the proximal end with the small serrefine.

Insert the left forefinger under the artery and, with the dorsum of the vessel lying on the finger, make a small upward triangular incision by snipping with the fine sharp scissors. The cut thus made should be about half the width of the blood vessel. Release the serrefine for a moment to be sure the blood will flow freely through the cut in the blood vessel, wipe with a sponge moistened in Ringer solution, and insert the cannula into the artery in the direction of the heart. After removing the finger from beneath the vessel, tie the cannula in place or secure it with a second small serrefine. Then allow the artery to return as far as possible to its normal position.

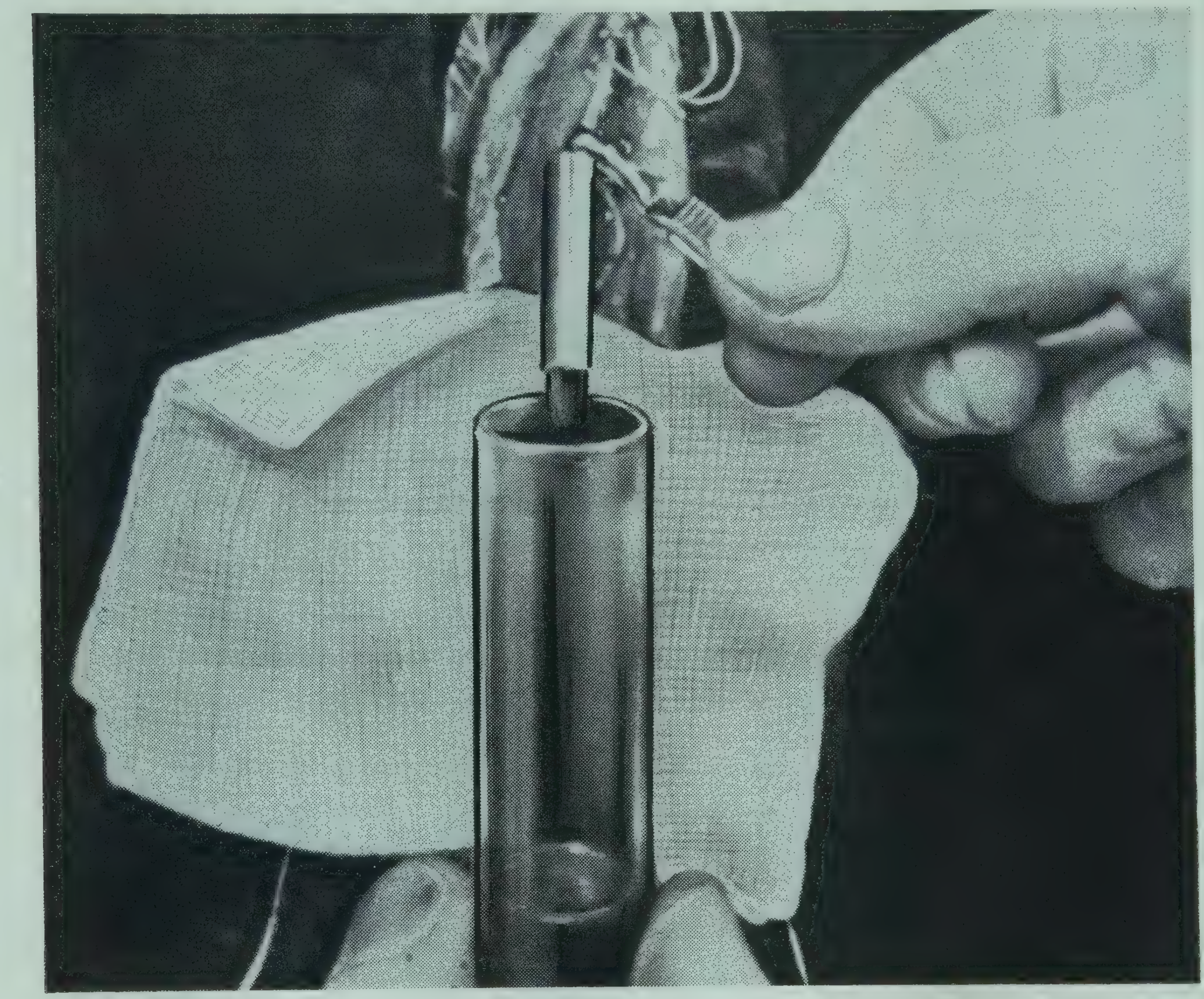


FIG. 17. Neck of fowl with blood flowing through cannula inserted into carotid artery.

Remove the serrefine clamping the artery, discard the first few drops of blood, then direct the flow so that the blood will fall straight to the bottom of the paraffined tube, avoiding drops or splashes on the edge (Fig. 17). Let the blood flow until the tube is a little more than half full when the tube should be exchanged for a fresh one. The assistant should immediately cork the first tube, and place it in a vertical position in the bowl of cracked ice. The corks must be guarded from contamination while they are out of the tubes.

Pressure of the operator's finger on the artery above the cannula will halt the flow of blood while the tubes are being exchanged. This is preferable to clamping each time with a serrefine, which might injure the vessel and produce an internal clot. The whole procedure should be done with as little handling of the artery as possible. Olive oil or yellow vaseline may be used to protect the artery from drying during the bleeding.

If the animal is to be bled to death, usually as much as 150 cc. may be obtained; but, if it is to be saved for future use, not more than about 100 cc. should be taken. Naturally the amount will depend on the size of the bird. After the bleeding is finished, ligate both sides of the incision firmly. Then cut the vessel through between the ligatures, because this aids in the speedy establishment of supplementary circulation. Wash off any blood and sew up the skin. A chicken recuperates immediately and should be given food and water at once.

CENTRIFUGING THE BLOOD

The tubes of blood, which have been standing in chopped ice, are placed in the chilled carriers, carefully balanced and centrifuged at about 2500 revolutions per minute for 8 to 10 minutes.

While the blood is being centrifuged, sterile pipettes for withdrawing the plasma should be made ready.

After centrifugation, the plasma will appear as a clear, transparent, straw colored, supernatant fluid. The red cells will be at the bottom of the tube, and a thin layer of leucocytes (the buffy coat) will be in between the red cells and the plasma. The plasma should immediately be pipetted into chilled paraffin tubes. A small quantity, about 5 cc., in each of several tubes is preferable so that if one tube is contaminated or broken, a large supply is not lost. When opened to receive the plasma, the tubes and the corks should be flamed and the tubes held at an angle to prevent dust falling into them. Care must be taken not to disturb the layers of blood cells when withdrawing the plasma, as broken down red cells have an inhibiting effect on cultured cells. The tubes of plasma are then stored in the refrigerator.

Some workers prefer ampules for storing media. When these are used, care must be exercised in sealing to avoid heating the contents.

WING BLEEDING

Noncoagulated plasma is less easy to obtain by wing or heart puncture, therefore it is advisable to use a small amount of heparin for these methods. About 0.5 cc. of a 1-5000 heparin solution drawn into the syringe before withdrawing the blood is sufficient to prevent coagulation of 10 cc. of chicken blood. Rinsing the syringe and needle with sterile olive or mineral oil will also aid in preventing coagulation.

Use a 10 or 20 cc. syringe and a 25 mm., 22 gauge needle.

Place the fowl gently on its back, tie the legs and one wing to the board, as for carotid bleeding. The wing to be bled should be placed *on* the sandbag instead of *under* it. A black cloth thrown over the bird's head, or a black mask covering its eyes, will keep it quiet for the short time needed. A comparatively feather-free area will be seen on the under surface of the wing close to the body. Remove the few feathers present, and a good sized vein will be visible through the skin, running parallel with the humerus. Wash the skin with iodine and alcohol and insert the needle through the skin into the vein, toward the wing tip against the direction of the flow. The gentle withdrawing of the plunger will fill the syringe with blood. Stop the bleeding by pressure, and centrifuge the blood immediately.

HEART PUNCTURE

Lewis (1928) uses a 2-inch, 20-gauge hypodermic needle on a 10 cc. syringe (Fig. 18).

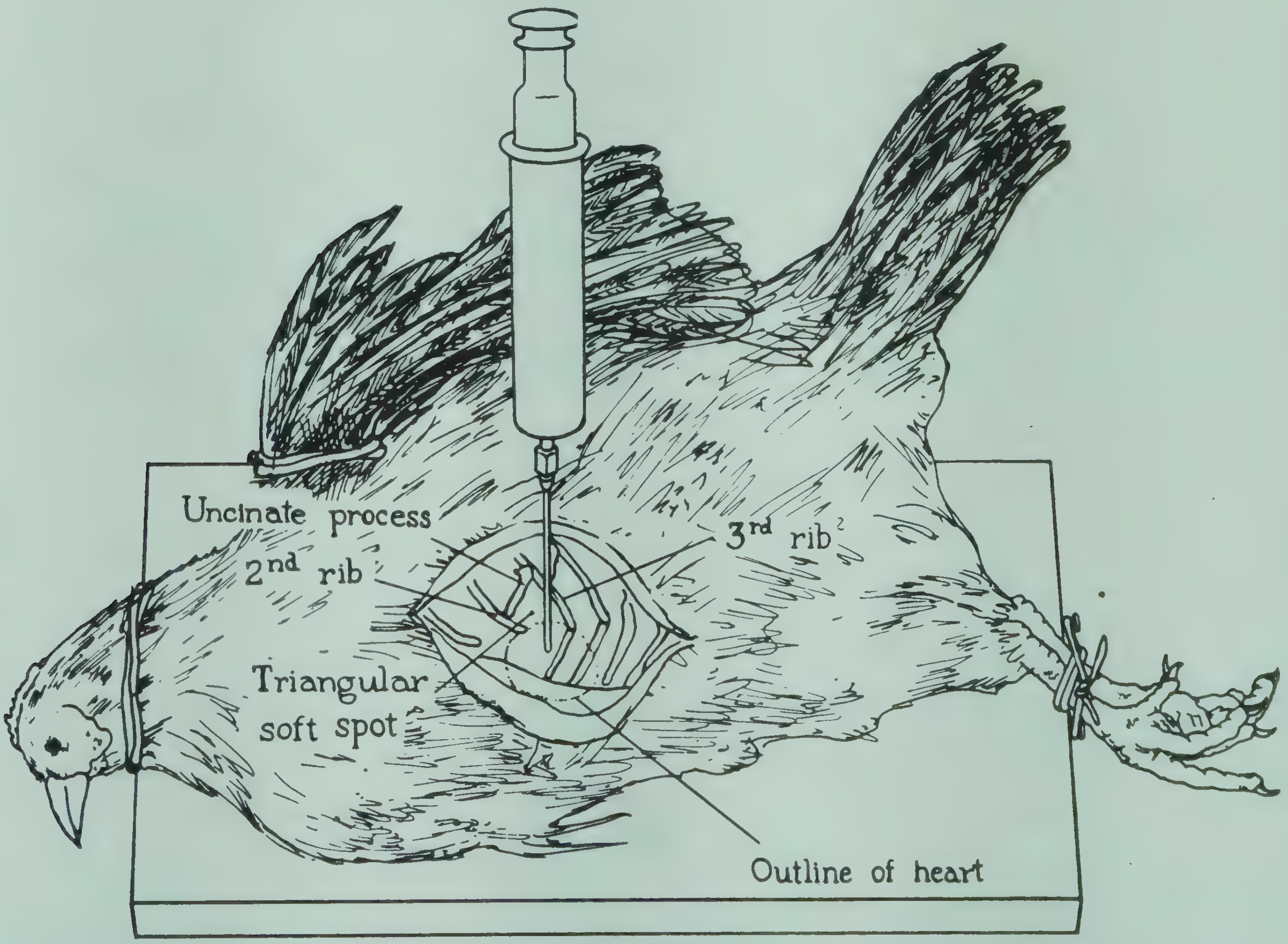


FIG. 18. Diagram of chicken tied to board for heart puncture (by R. Buchsbaum after Lewis).

The bird is placed gently on its left side, the right wing folded in its natural position beneath it. The feet are tied together to the foot of the board, the neck gently fastened to a hook at the head of the board, and the left wing tied back to the side of the board.

After dampening the feathers around the cardiac region with alcohol, the larger ones may be pressed out of the way and the smaller ones plucked. A large vein will then be seen running from sternum to shoulder. Posterior to this vein and bounded by the second and third vertebral ribs and the uncinate process of the second rib, a soft triangular area may be palpated. Using this as a landmark, a second soft spot through which the needle should be inserted can be located. This is also posterior to the vein and just ventral to the sternal end of the second rib and anterior to the sternal margin of the third rib. It usually lies just under the dorsal edge of the breast muscle.

The skin should be painted with iodine, washed with alcohol and allowed to dry.

Insert about half the length of the 2-inch needle. This should be done slowly so that the pulsation of the heart can be felt and the needle not inserted too far. Gentle withdrawing of the plunger will fill the syringe with blood, which should be centrifuged at once and stored in the refrigerator.

DESICCATED PLASMA

Desiccated plasma is obtainable in 5 cc. vials to which 4.5 cc. (90%) of distilled water is added. The accompanying distilled water prepared for reconstitution is partially saturated with CO_2 to compensate for CO_2 loss during processing. The Tissue Culture Association reports that if ordinary distilled water is used the pH of the plasma will be unduly alkaline, varying from 8.5 to 9.0. The water should be introduced into the vial gently and the contents dissolved by rotation, not by shaking of the vial. This product acts perfectly when freshly reconstituted, with a progressive retardation of coagulation time on storage in the liquid state. It will keep indefinitely in the desiccated form.

BLEEDING MAMMALS

Most workers use mammalian sera (in conjunction with fowl plasma) and therefore do not find it necessary to obtain uncoagulated mammalian plasma. However, if it is desired, it is necessary to use an anticoagulant. Of these heparin is the least toxic and, like most chemicals, retains its potency longer when stored in concentrated solution. It is prepared in Ringer solution (since Tyrode may not be autoclaved) in a concentration of 1-1000, autoclaved and, when needed, diluted with Tyrode to 1-5000. Different samples of heparin vary considerably in potency and toxicity and each lot should be tested before large scale use. We have found Liquaemin (Organon) most dependable. It contains a trace of phenol but not enough to produce observable deleterious effects on the cells. One part of 1-5000 heparin solution to five parts of blood is usually sufficient, but stronger concentrations may be used if necessary. Put the heparin solution in the tube into which the blood is to flow and mix the blood and heparin well with a sterile pipette before centrifugation. Gey* makes up his heparin 10 mg. to 1 cc. of normal saline and adds enough to dog or chicken blood to make the total concentration of 1-200 heparin in the blood. Sodium citrate which has been used in the past as an anticoagulant is less desirable than heparin for use in tissue culture media.

Rabbits and guinea pigs may be readily bled from the carotid artery or from the heart.

The carotid artery bleeding of rabbits and guinea pigs is done exactly as described for chickens, but guinea pigs need twice as much heparin as other mammals.

To bleed a small animal (such as a rat) from the heart use a 5 or 10 cc. Luer syringe (depending on the size of the animal), fitted with a sharp, 25-mm. 22-gauge needle. Anesthetize the animal by placing it under a bell jar in which cotton moistened with ether is present. When anesthetized, the animal is fastened spread-eagle fashion to a board, and the hair removed from the thorax by shaving. Swab with iodine and alcohol. The heart can be felt on palpation with very light pressure and the point of entry is that of the most marked beating. Insert the sharp needle into the ventricle with a quick puncture, taking care not to allow the needle to pass through and touch the posterior wall. Usually the flow of the blood will push back the plunger of the syringe. Do not use force in withdrawing it or the suction will draw the wall of the ventricle against the needle and occlude the opening.

Human blood is usually obtained from the median, basilic vein of the arm. The donor should be comfortably seated facing a good light. A tourniquet or a piece of muslin bandage should be tied just above the biceps and the area over the selected blood vessel cleansed first with soap and water, then with alcohol. A long, sharp needle of large caliber is inserted obliquely upward into the vein at the bend of the elbow. After discarding the first few drops which come through the needle, the blood is allowed to flow into a tube. When sufficient blood has been obtained, remove the tourniquet, place a dry gauze sponge over the puncture and bandage firmly—but not so tightly as to interfere with the circulation. A variation of the above method is to attach the needle to a 20 cc. Luer syringe rinsed in sterile olive oil, and draw the blood gently into the syringe instead of allowing it to drop into the tube.

---

* Personal communication.

By using a vein in the leg this method is easily adapted to the bleeding of dogs and other large animals, e.g., goats, sheep, pigs, etc.

BLEEDING OF COLD-BLOODED ANIMALS

Many cold-blooded organisms may be bled by practically the same methods as those used for animals.

In bleeding *frogs* we have found that all extraneous bleeding may be avoided and more blood and nonhemolyzed plasma and serum obtained by the method used by Hsiao in this laboratory. A large bull frog (or a *Rana pipiens*) is pithed and tied to a frog board. The abdominal skin is cut and reflected, the mediastinum is split up to and through the clavicular junction exposing the thoracic cavity. The pericardial sac is opened exposing the heart, bulbus arteriosus and the proximal portions of the two systemic arteries. Three ligatures are placed around them, one ligature is placed loosely around the right systemic artery and the other two around the left in the positions shown in Fig 19. The distal ligature of the left is now tightened to occlude blood flow. The same artery is then clamped with a small serrefine proximal to ligature 2, and a V shaped snip made in the artery with fine pointed scissors between the serrefine and ligature 3. Thereupon a glass or metal cannula, sterilized in oil, is

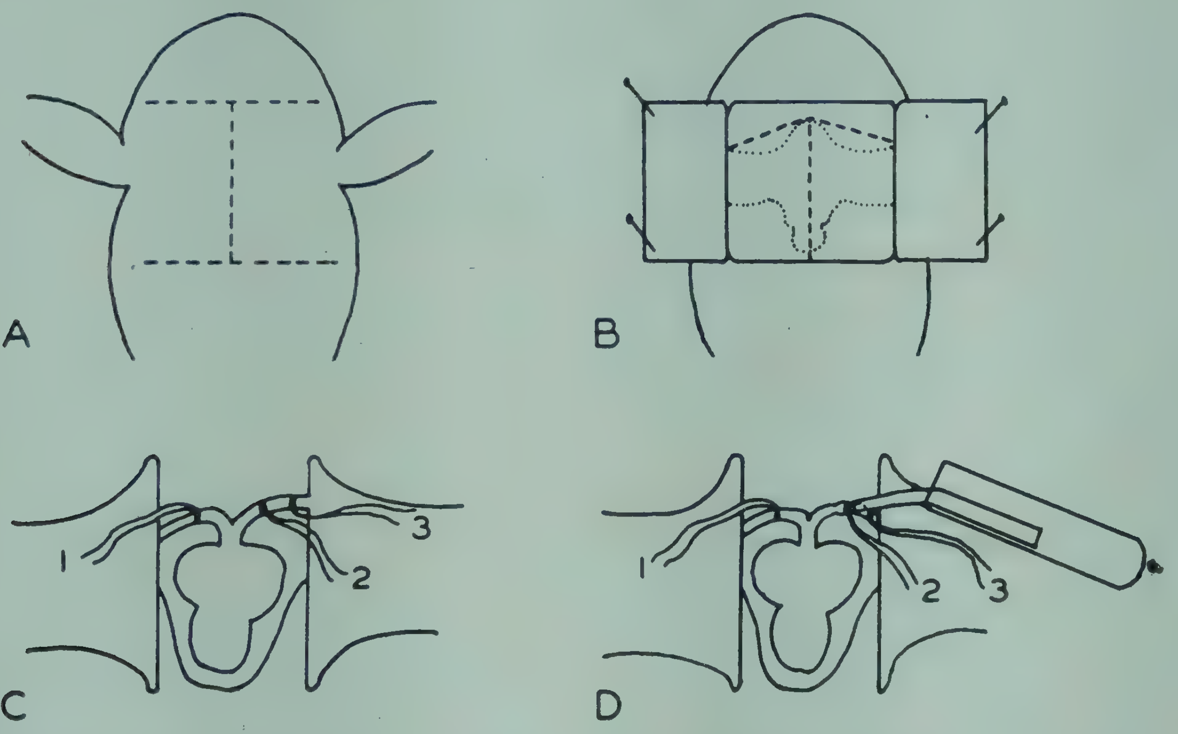


FIG. 19. Diagram of bleeding of frog from systemic artery. (Personal courtesy S. C. Hsiao.)

TECHNOLOGICAL RESEARCH
LIBRARY

inserted centrad into the vessel and tied in place with ligature 2. Ligature 1 on the right vessel is now tightened and the blood is thus directed from the heart entirely toward the left. The release of the clamped serrefine permits flow of the blood into a chilled paraffin tube. The frog board may be tilted so that gravity will aid the flow. The blood is then centrifuged as usual. About 1 cc. of clear supernatant plasma will be obtained from a small frog and 2 to 3 cc. from a bull frog.

Salamanders may be quieted for bleeding by adding a narcotic such as MS222, 1-1000 dilution to the water 3 to 5 minutes before bleeding, or by chilling with ice. Special care is required to prevent the heavily infected skin exudate from entering the pericardium. After the cutaneous incision is made, we have found it helpful to deflect the skin by means of curved hooks attached to weights which are hung over the edge of the operating board and table. It is then a simple matter to locate and puncture the heart or cannulate the aorta. Charipper and Dawson (1928) recommend using a fine oiled needle as a cannula, inserted into the afferent vessel of the gill.

Carp, dogfish, squid and *lobsters* may be bled by heart puncture. The handling of lobsters is facilitated by wrapping them in a towel or piece of cheesecloth.

To bleed *turtles* a "window" is cut through the sternal plate overlying the heart. This may be done by drilling holes with a trephine in the four corners of a rectangle 25 to 30 mm. square, after which the intervening shell is cut away with bone forceps. The exposed pericardium should be cut through and the edges held with artery clamps to prevent the large amount of body fluid present from entering the pericardial sac. Blood may then be easily withdrawn from the heart into a syringe.

SERUM

The general use of plasma as an integral part of most clotted culture media is paralleled by the use of serum as a component of fluid media. Because of the tough fibrin present in fowl plasma, it is necessary to grind the clotted plasma to obtain serum from it. There are various devices for this purpose now on the market but for occasional use and for small quantities it may be prepared as follows:

Add a few drops of tissue extract to a tube of plasma and allow the tube to stand at room temperature for about 10 minutes or until completely coagulated. Flame the top of the tube and loosen the clotted plasma from the sides of the tube with a sterile knife or spatula and empty into a mortar. Add a small amount of sterilized sand and grind the mixture with a pestle. Replace in a tube and centrifuge thoroughly. Clear serum will be obtained from the upper part of the tube. Sterile materials must be used and sterile technique carefully observed throughout.

Because *mammalian* plasma contains less fibrin than that of fowls, grinding with sand is not necessary. Serum can readily be obtained from clotted mammalian plasma by stirring with a glass rod or pipette and then centrifuging it. If the blood clots in the tube before the plasma can be removed, it should be placed in the refrigerator until the serum separates. It is then centrifuged, after which the serum is removed with a pipette.

For many years it was considered necessary to provide homologous sera for cultured cells, but more recent experimentation has proved that most of the protein required for growth and protection is nonspecific. When more convenient, heterologous sera may be used with no fear of untoward results. For example, Earle routinely uses horse* serum for all types of tissue. In culturing some mammalian tumors where there is excessive liquefaction of the clotted medium, horse serum is an advantage as it tends to inhibit the proteolytic action. It is a convenient medium since it is usually available in large quantities, is rarely hemolyzed, and is unchanged by many months of storage at 0 to 5°C. By heating in a water bath at 56° for ½ hour, sufficient of the specific proteins (such as complement) are denatured to the extent that horse serum may be used as a constituent of fluid media for almost any species. In this laboratory, with appropriate dilution for isotonicity we use it even for cold-blooded forms. If obtained commercially, it should be checked for the presence of preservative which is frequently used.

Human placental cord serum is also highly recommended. Introduced by Gey and Gey (1936) it has been routinely used in many hospital-connected laboratories. Since it is now obtainable in desiccated form its more general use will be possible.

Any serum which is suspected of possible contamination should be passed through a Selas filter before the toxic products of bacterial growth have the opportunity to render the serum unfit for culture media. The Boerner Centrifugal Filter adapted for use in the International Centrifuge is convenient for small quantities of serum. It utilizes centrifugal force instead of suction or pressure to force the serum through a Seitz filter disc.

---

* Now obtainable in desiccated form.

CHAPTER VII

PREPARATION OF TISSUE EXTRACTS

Many years ago Carrel (1912) discovered that the addition of aqueous extracts of embryos to a plasma culture medium renders possible indefinite proliferation of cultured cells. Since that time tissue extracts have been almost universally used as a component of the medium when proliferation of cells is the desired goal. Tissue extracts also aid in the clotting of plasma and the formation of a firm coagulum which supports the cells. The dual properties of stimulation of cellular proliferation and of clotting have been found to be nonspecific, so that the majority of workers use the easily obtainable young chick embryos for this purpose. Mammalian embryos, if more convenient, serve equally well. For instance, Gey, Hanks and Barrett (1948) use young beef embryos obtained aseptically from a slaughter house, while some workers prefer those of mouse, rat, guinea pig, etc. In the cultivation of tissues from cold-blooded organisms, we have found extract of tadpoles highly effective. Others, notably Hoffman and Doljanski (1939), Doljanski and Hoffman (1943), Doljanski and Werner (1945), have substituted extracts of various adult tissues for those of embryos and have cultivated fibroblasts "for long periods" by this method while Earle (1937) and others have found that tumor extracts activate some types of cells.

For chick embryos arrangements may be made for a regular supply of fertile eggs to be incubated in the laboratory. Two dozen eggs weekly, from a good strain, should be sufficient for a small unit. They should be marked with the date before being placed in the incubator, where they are incubated at 38°C. and turned twice daily to prevent the embryo from adhering to the shell. In New York sufficient moisture is present in the air, but in dry localities a certain amount of moisture must be maintained by keeping a small container of water in the incubator.

It is easy to work out a schedule so that embryos of the required age are ready when needed. Embryos 8- to 10-days old are best for tissue juice. Younger embryos are wastefully small, and older ones furnish juice which is less activating; also, at about 12 days, the presence of developing feathers renders the resulting extract cloudy. A turbid extract deteriorates more rapidly than a clear one.

It is a good plan to candle the eggs after 5- or 6-days incubation and discard any which are not developing normally. This prevents noxious gases from dead embryos from poisoning the remainder of the eggs. Frequently, candling apparatus is included when an egg incubator is purchased, but if not, one can easily be arranged. Place an electric bulb (75 or 100 watts) in a box painted black or covered with black cloth and cut a window in the box to accommodate an egg; when tested with this apparatus in a dark room or cupboard, the developing embryo and blood vessels are easily distinguished.

Although embryo extract may be used for several days or even a week after its preparation, it is better, as a routine, to make it fresh each culture day. Experience has shown that the growth-promoting substances gradually deteriorate in stored extracts. More nearly uniform conditions are obtained in the culture medium if the extract is always used at its optimum activity.

There are various methods by which extracts may be prepared. The following is a simple method for the preparation of a small amount without involving the use of special equipment.

PREPARATION OF A SMALL AMOUNT

MATERIALS REQUIRED

Not sterilized
- Granite bowl
- Towel
- Instrument for breaking eggs
- Incubated eggs
- Microburner

Sterilized
- Table cover
- Box of instruments for extract
- Pipettes (large)
- Rubber nipples
- Package of sponges
- Watch crystals
- Small tubes (9 × 90 mm.)
- Tyrode solution
- Pipette rack and tubes
- Petri dish (150 mm.)

Before the sterile materials are opened, the culture room should be aired, then the windows and doors closed, and the room cleaned. The operator should cover his hair, put on his mask and, after thoroughly washing his hands, spread the sterile table cover and arrange the needed supplies. Put the nonsterile articles on the assistant's table to the right. If the nipples have been sterilized in water, they should be squeezed dry on sterile sponges. See that no water remains within the nipples to run down into the pipettes. Attach nipples to several pipettes and place them n tubes in the metal rack. Carefully flame the neck of the flask containng the stock supply of Tyrode, discard the first few drops passing over the lip of the flask, and pour the desired amount, 50 to 100 cc., into a 125 cc. Erlenmeyer flask. The pH of the Tyrode should be checked at this time since even under the best conditions it may change. If phenol red is present the pH may be determined at a glance and should be between 7.6 to 7.8. Methods of adjusting it will be found in Chapter V.

The operator should sit on a comfortable stool in front of the table and place the bowl on a towel in his lap. Place a watch crystal in the lower section of a 150 mm. Petri dish and cover it with the upper portion. Hold an egg by the pointed end in the left hand and with an instrument such as the handle of a scalpel, crack the egg lightly in the middle from the side, break the shell in two, letting the upper half of the shell drop into the bowl. Take care not to touch with the fingers the embryo and the yolk remaining in the other portion still held in the left hand. With the straight scissors snip the membranous sac enclosing the embryo. Slip the curved forceps under its neck, lift the embryo and drop it into the watch crystal.

When the desired number of embryos have been obtained wash them with Tyrode to remove any traces of blood for broken down red blood cells inhibit growth. Then, holding the edge of the crystal firmly with the thumb and finger of the left hand, keep chopping the embryos with the curved scissors until they are reduced to a fine pulp. Add an equal volume of Tyrode solution, usually about 2 cc. for each 9-day embryo, and mix by drawing several times into a wide mouthed pipette before transferring to narrow tubes (9 × 90 mm.). Allow to stand for about one-half hour before centrifuging for 10 minutes at 2000 r.p.m. Withdraw the supernatant fluid and recentrifuge.

Mammalian extract may be made in the same way. The placenta should be discarded and the embryos washed free of blood. Embryos in the first half of pregnancy are preferred as they produce a clearer extract than older embryos. A cloudy extract may be clarified by inserting a pledget of cotton in the test tube below the stopper. During centrifugation the pledget is driven centrifically and thus acts as a sliding filter. This device, recognized in bacteriological technique, is being used in tissue culture by Gey, Hanks and Barrett (1948).

For *tadpole extract* select tadpoles 2 to 2.5 cm. in length and immerse them in 1-10,000 sodium merthiolate solution for 3 to 5 minutes. Rinse thoroughly in sterile Holtfreter solution, remove the gut, rinse in penicillin 100 u/cc. and proceed as for chick embryos. As an added precaution against contamination, penicillin 100 u/cc. may be added to the pulp suspension before centrifugation without adverse effects on the cells of the cultures.

Various mechanical devices have been introduced to replace the watch crystal method. Earle (1932) forces young embryos through a monel metal screen inserted in the base of a 20 cc. syringe, by pressure on the plunger. This is an excellent method for a few young embryos, but is not suitable for more developed ones as sufficient pressure cannot be exerted without danger of breaking the syringe. Carpenter (1947) has devised a sturdier mincer of the same type which is made entirely of monel metal and may therefore be used for any type of tissue.

PREPARATION OF LARGE AMOUNTS

If a large quantity of extract is required, as for example, to feed numerous flasks or tube cultures, it is advisable to use a grinding machine instead of scissors for chopping. The La Tapie apparatus or a Rosenow crusher are suitable for this purpose. Since they have no rubber washers, they may be sterilized by dry heat. Moreover, they are carefully fitted to prevent leakage which would be a source of contamination.

Various other types are also available, such as the Tenbrock grinder, which comes in several sizes, or Potter's modification of it. Gey uses a Whiz-Mix, Parker a specially designed tube fitted with a glass plunger having teeth for crushing and electrically operated somewhat like the Waring blender. The Waring blender is not recommended except for large amounts of fibrous or osseous tissue. It mashes instead of cutting or separating the cells and a turbid extract results which contains substances that have a deleterious effect on cultured cells.

It has been shown by Hetherington (1944) that extracts (like plasma and serum) may be lyophilized and stored indefinitely in ampules in the desiccated state. He reported that after 2 years of such storage, the addition of water reconstituted the extract into one quite as effective as one freshly made. Lyophilized chick embryo extract, approved by the Tissue Culture Association, is now obtainable commercially in 5 cc. vials. To prepare this for use, add 4.5 cc. of redistilled sterile water to the vial. Remove the suspension with a wide-mouthed pipette to a 50 cc. centrifuge tube. To make an extract of strength comparable to those freshly made by the above methods, add 20 cc. of physiological salt solution to the suspension. Let stand at room temperature for about 1 hour, rotating occasionally, then centrifuge for 10 minutes at 2000 r.p.m.

For *adult tissues* it is best to use some type of grinder and the proportion of salt solution to pulp should be increased to about five parts of Tyrode solution to one of pulp to compensate for the smaller amount of water present in adult tissues. The extract should be allowed to stand for about 2 hours before centrifugation.

Extracts of *inflamed tissues* have been found to stimulate cell growth. Such tissues may be produced experimentally by injecting under the skin of adult rabbits about 2 g. of sterile infusorial earth (Kieselguhr) suspended in sterile saline. After about 48 hours, the inflamed tissue with the infusorial earth is cut out, chopped in a tissue crusher and treated as any other adult tissue.

Bone marrow and blood leucocytes provide growth-promoting extracts. For the first, crush the larger bones (femur, etc.) of a young adult animal with a bone crusher, add Tyrode, and centrifuge. For the blood leucocytes, centrifuge the blood as soon as obtained, pipette off the plasma, and remove to a small tube the film of white cells (buffy coat) lying between the red cells and the plasma. Mix with Tyrode and centrifuge. If the buffy coat is coagulated, it can be removed as a whole, placed in a watch crystal and chopped with scissors.

Extracts of individual organs, such as spleen, brain, kidney, etc., which may be desired for special experimental purposes, are made as any other tissue extract, the amount of tissue used determining the method.

The whole procedure, by whatever method, requires the utmost care, since tissue juice is a medium for pronounced bacterial growth and faulty preparation of embryo extract is a frequent source of contamination. If, for example, a slip is made when opening an egg, never "take a chance." Discard it and, since the crystal must be held by the fingers, care must be taken not to allow either the embryo or the Tyrode to touch its edge. The tube in which the juice is centrifuged being narrow, the method of transfer also must be guardedly watched. Should there be any reason to question the sterile condition of the extract, bacteriological cultures should be made as described in Chapter V.

No attempt should be made to sterilize contaminated extract. Passing it through a filter removes a large percentage of the growth-promoting substances, while the toxins produced by the bacteria are left in. Heating destroys the growth-promoting substances and ultraviolet radiation reduces its ability to coagulate plasma.

Some workers routinely freeze the freshly prepared extracts either in a "deep freeze" or in CO_2 snow. This serves the dual purpose of destruction of occasional cells which may inadvertently have been carried over in withdrawal of the supernate, and of convenient storage. In the frozen state the usual progressive loss of potency is held at a minimum.

CHAPTER VIII

ARTIFICIAL MEDIA

Since tissue culture became an accepted method of biological experimentation, investigators have been seeking the elusive "perfect medium" for growth outside the body. Carrel's (1912) basic plasma and extract medium assuredly promotes indefinite growth, but the importance of the development of a medium of known composition which may be repeated at will, has engrossed the attention of an increasing number of investigators. The Lewises (1911a) pioneered in the use of artificial media with agar and bouillon, later with physiological salt solutions (1911b) to which were added glucose and peptone. As a basis for the preparation of an adequate synthetic medium, many attempts have been made to analyze media known to support growth such as serum and tissue extracts. Baker and Carrel (1926a,b) made careful analyses of chick embryo extract and tested the isolated components to produce a solution comparable in activity to the whole extract. The tests were made with cultures in a medium containing the usual amount of plasma. Many activating combinations were developed, some of which were specifically favorable for a certain type of cell, but none equalled the whole extract in ability to stimulate and maintain growth. They came to the conclusion that unidentifiable proteinases in the extract acted on plasma to produce protein break-down products utilized by the cells.

Vogelaar and Erlichman (1933) also developed a solution to replace embryo extract containing Witte's peptone, glucose, insulin, hemin, thyroxine and cystine which, combined with plasma, was adequate to support the cells for a limited time. Baker (1935) increased the effectiveness of this solution by the addition of various substances including vitamins A, D, and C.

Fischer (1941a,b) (1948a,b), Fischer and Astrup (1942, 1943), Fischer et al. (1948) have done extensive work on the nature of the substances required for permanent maintenance of cells. They point out that cells *in vitro* subsist under very different conditions from those *in vivo* and that specific nutrients must be made available for the life of cells outside the body. By many experiments involving dialysis, fractionation, filtration and heating, these investigators came to the conclusion that a satisfactory tissue culture medium must contain two components, one a high molecular weight growth promotor of a protein nature found in embryo extract and, two, an accessory substance of low molecular weight. They then attempted to compose a synthetic medium meeting these requirements. A resulting solution termed Fischer V614* is a substitute only for the accessory growth substance and is made according to the following formula:

	Mg. per liter
NaCl	7500
KCl	200
$CaCl_2$	200
Na_2HPO_4	50
$NaHCO_3$	1000
$MgCl_2$	100
Glucose	2000
Ca-hexosediphosphate	200
Cystine	10
Glutathione	10
L(—)-Lysine, 2HCl	30
L(—)-Histidine, 2HCl	10
L(—)-Arginine	4
D,L-Valine	28
L(—)-Leucine	18
D,L-Isoleucine	20
D,L-Threonine	24
D,L-Phenylalanine	14
L(—)-Trytophan	4
Glutamine	250

Simms and Stillman (1937a,b,c), Simms and Sanders (1942), Simms, Parshley and Pitt (1947) made extensive studies of the growth requirements of adult cells in tissue culture by exposing the cells to various fractions that they obtained from adult serum and tissue extracts. These fractions were designated as follows: Factor A, which stimulates dormant adult cells; factor B ("lipfanogen"), which reversibly causes fat deposition in the cells; factor anti-B ("antilipfanogen"), which is antagonistic to B; factor C, which causes cellular degeneration; and factor D, which produces cohesiveness of fibroblasts. They prepared a serum ultrafiltrate from ox blood which is now commercially available, designated as UF. Together with UF, Simms' physiological salt solution, X7, is also available. For use as culture medium they recommend diluting UF with X7 in proportions of 1 to 2. This mixture is available as UF_3. This solution has been found of special value in preventing fat deposition in cells from adult tissues. While it is not a complete medium for prolonged cultivation of tissue cells, it is excellent when used in combination with serum or plasma and very efficacious as the basis of a medium for the cultivation of many viruses.

White (1946) has attempted to combine the substances necessary for growth in one mixture, made up to include several inorganic salts, eleven amino acids, glutathione, dextrose, vitamin A, vitamin C and all the known B vitamins. For testing the effectiveness of his formula, he used roller tubes into which he introduced a brei of embryonic muscle tissue, allowed it to dry on the surface of the glass and added his nutrient fluid. Muscular contraction, which he used as his criterion, was maintained for periods up to 50 days. Several investigators, Fischer et al (1948), Jensen (1948) and Jacoby and Darke (1948) have questioned White's methods, suggesting that the embryonic tissue contained unknown substances which would, by the circulation of the supernate, be incorporated into it. Jacoby and Darke (1948) used White's medium on pure cultures of monocytes in Carrel flasks where no traces of tissue juices or serum remained and report rapid and irreversible deterioration of the cells in a few days.

A variant of the above methods for identifying essential ingredients of the medium has been to add to whole plasma specific substances such as ascorbic acid, Chambers and Cameron (1943), cortical compounds of adrenal gland, Chambers and Cameron (1944), creatin, Caspé and Cameron (1946), diaphorase and coenzyme, Caspé and Cameron (1944), egg albumen, and peptic digests of albumin and fibrin and various other substances, Osgood and Brownlee (1937).

The production of lyophilized culture media already mentioned is a step in the direction of a standardized medium as also is the use of fibrinogen and thrombin to replace plasma for cell support. Several investigators have reported favorably on its use; Porter and Hawn (1947) and Favata (1947) give descriptions of their methods.

The *fibrinogen* used by Porter is obtained from bovine plasma (Fraction I, Armour & Co.). Of this material 40% is citrate, which Porter has not found harmful. It is prepared for the culture medium as follows:

Make a 0.5% stock solution of fibrinogen in Tyrode solution (taking into account the citrate, this would be 1.2 g. to 100 cc.). Fibrinogen dissolves slowly. To aid this process the container may be rotated, but never shaken. Pass the solution by pressure through a Seitz filter and store at 0°C. The solution will remain in satisfactory condition for from 7 to 8 weeks.

The *thrombin,* also of bovine origin, is obtainable in vials containing 5000 N.I.H. Units (Parke, Davis Co.) and is accompanied by 5 cc. of diluent. It dissolves readily. Dilute the thrombin solution to 10 to 20 units per cc. in Tyrode solution so that one drop will contain 0.5 to 1 unit. Thrombin is much less stable than fibrinogen and must be kept at dry ice temperature (–50 to –70°C.). It loses nine tenths of its potency at 0°C.

The pH of the mixture of the fibrinogen and thrombin is important in relation to the transparency of the clot. At pH 6.5 to 7 the clot becomes opaque due to the large size of the fibers. At 7.5 and higher the fibers are fine and the clot transparent. After clotting has taken place, the pH of the supernatant fluid or gaseous medium may be varied without loss of transparency. Such a clot remains firm in the presence of chick cells but mammalian cells cause some liquefaction. For methods of preparing the cultures in this medium see Chapter XI (for slide cultures) and Chapter XII (for flask cultures).

Fischer (1949) reports on the development of a trypsin inhibitor from soybeans. He reports that in the cultivation of Rous chicken sarcoma, the inclusion of a small amount of the inhibitor in the coagulated culture medium completely inhibited liquefaction of the clot, which was extensive in the controls. The growth of the cells was slowed in the presence of the soybean inhibitor, affecting their proliferation less when introduced into the supernatant fluid instead of into the clot.

In recapitulation, the aim of the earlier investigators was primarily to induce growth. Subsequently analyses were made of the ingredients of plasma and tissue extracts with results which are still in process of further investigation. No one has as yet been able to produce an artificial medium as efficacious as the whole plasma and tissue extract medium. In other words, the assembly of the products of fractionation, together with the addition of presumably essential constituents have thus far failed to produce a thoroughly satisfactory growth medium. The analytical work is being continued and is of extreme importance not only for an understanding of the theoretical problems concerning cellular growth, but also to provide a standard means of reproducing a given environment at will.

CHAPTER IX

PREPARATION OF TISSUES

There are two general types of tissue cultures, mixed cultures composed of a variety of cell types and pure cultures composed of one type of cell only. The mixed cultures, with a variety of cells, more nearly reproduce the conditions obtaining *in vivo* and are by far the most generally used.

The preparation of all tissues for cultivation requires not only care to avoid bacterial contamination, but precautions to prevent drying and gentle handling to prevent injury to the cells. As a general rule it is wise practice for a beginner to confine his first efforts to the cultivation of chick embryonic tissue. This is easily obtained in a sterile condition and the technique is simple. The tissue grows well and the danger of contamination is less than when mammalian tissue is used, because chicken plasma (unlike mammalian plasma) seems, even *in vitro,* to retain a certain resistance to bacteria. From the cultivation of chick tissue the operator will obtain an idea of the appearance and comparative rate of growth of the various type of cells. When, with experience, a certain proficiency has been attained, other types of tissue can be successfully cultured.

MATERIALS REQUIRED FOR PREPARATION OF TISSUES AND FOR SLIDE CULTURES

Nonsterile

- Table board
- Bakelite square
- Microburner
- Dissecting needle holder
- Bowl of cracked ice
- Empty bowl for discards
- Flat enamel pan for discarded pipettes
- Paraffin in cup
- Electric plate or Bunsen burner
- Camel's-hair brush
- Labels (paper and adhesive)
- Incubator racks

Sterile

- Table cover (black sateen)
- Package of sponges
- Rubber nipples
- Pipette rack
- Glass tubes, 25 × 100, for holding pipettes in rack
- Pipettes (fine and coarse)
- Box of slides
- Coverslips (square, oblong, round)
- Mica coverslips
- Petri dishes (for covers) 100 mm.
- Box of instruments
- Yellow vaseline
- Strips of filter paper
- Erlenmeyer flasks 125 cc.
- Media (plasma, extract, serum, etc.)
- Ampule 1% phenol red

SETTING THE TABLE

The making of a culture is the culmination of all the work in the previous chapters. As it is a sterile operation, all the necessary precautions should be observed, with the room cleaned, etc. The room will be partially ready for culture work if the routine follows immediately after the making of embryo extract (see Chapter VII).

As usual, the nonsterile materials are placed on the assistant's table, and the sterile materials on the culture table (Fig. 20). The set-up for culture work is as follows:

The table board (Chapter III) should be placed in position on the culture table, and the sterile black sateen table cover laid over them.

Fresh sterile tubes should be placed in the pipette rack (those used in making extract having been discarded) and nipples attached to fresh sterile pipettes. The discarded pipettes are placed in cold water in the flat enamel pan provided for that purpose.

The boxes of depression slides, coverslips, Petri dishes, instruments, vaseline, etc., are then removed from their paper wrappings and arranged in a convenient position on the culture table. Everything must be kept covered except at those moments when an article is removed for immediate use.

EMBRYONIC TISSUES

After the embryo has been removed (as described in Chapter VII) it is washed free of blood and, in the case of mammalian embryos, the placenta removed. The desired tissue is then removed with an iridectomy forceps or a fine curved scissors, placed in an oval depression slide containing Tyrode, and rinsed to remove traces of blood.

Holding the fragment with a needle on the flat platform of the oval depression slide, cut it into pieces 1 to 2 mm. in size, bearing down with a keenly sharp-edged cataract knife. Avoid pulling or stretching. The tissue may be cut to any size or shape for special purposes, for instance, into strips such as Earle (1939) uses for some flask cultures. Return the pieces into the deep part of the depression slide where they will be covered with Tyrode and cover this with a Petri cover. Some workers use scissors for cutting the tissues because it is quicker, but difficulty is encountered in obtaining pieces of uniform size, and there is apt to be more cell injury. Others use two No. 7 blades, which have a straight cutting edge, fitted into Bard Parker handles. A knife is held in each hand, the blades placed close together on the tissue and a cut made with each knife simultaneously in opposite directions. This is satisfactory for fresh tissue but is less suit-

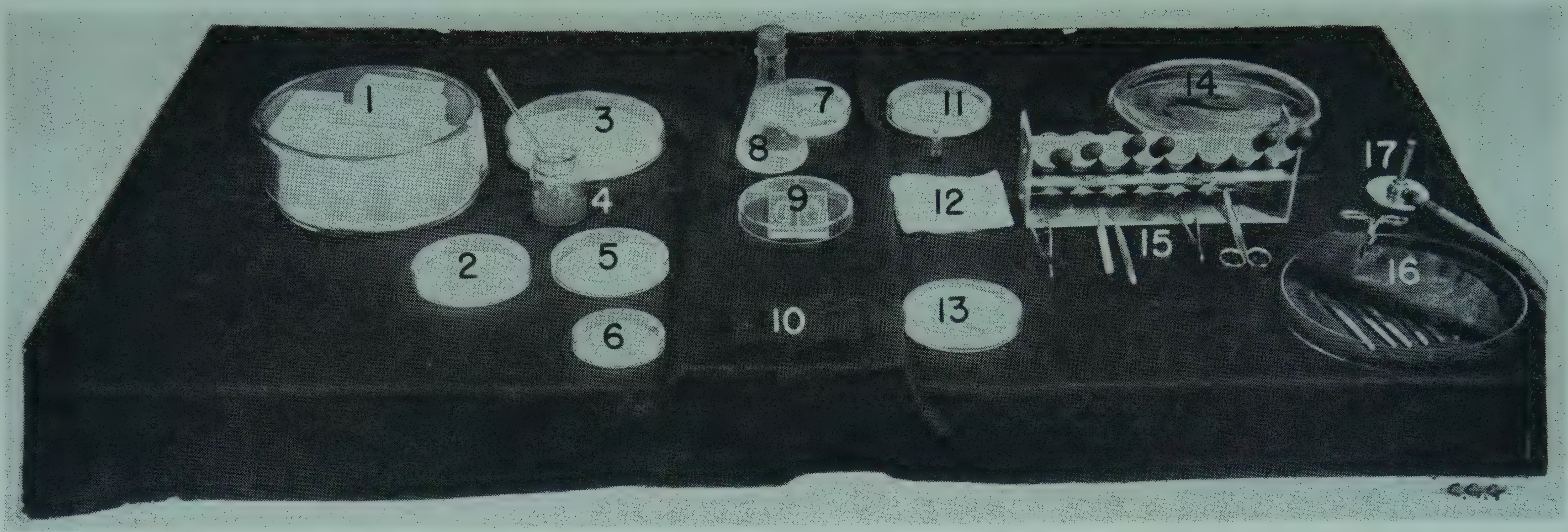


Fig. 20. Table top set-up for culturing. 1. Box of slides; 2, oblong coverslips; 3, watch crystals; 4, vaseline; 5, mica squares; 6, small circular coverslips; 7, blades for removing paraffin; 8, Tyrode solution; 9, oval depression slides for tissue; 10, block of bakelite; 11, strips of filter paper; 12, sponges; 13, dissecting needles; 14, cover for instruments; 15, metal pipette and instrument rack; 16, instruments; 17, microburner.

able for cutting the delicate new outgrowth of cultures to be transplanted.

Tissues cut sharp and clean produce better and more even growth than when torn. Teasing of the fragment may be desirable when the primary purpose is the study of an explant, e.g., of secreting kidney tubules. In this case capsular connective tissue and extraneous adherent materials such as gonad, etc., should be removed and the kidney tubules gently teased apart, not cut, with the *back* of the knife. By this means uninjured segments of the tubules may be well spread when placed in the culture medium. If growth of sheets of kidney epithelium is the primary purpose, the tissue is cut in the usual manner.

ADULT TISSUES

If adult tissues are to be used the danger of infection must be considered and care taken to retract the skin and, in case of viscera, to avoid puncturing the alimentary canal when making dissections.

Hanks* has found it possible to obtain sterile cultures of adult skin, usually from the rabbit. A selected area of skin is scrubbed with soap, shaved, and washed with alcohol. A pad of 2% iodine is placed on the area for 2 minutes, followed by alcohol. The concentration and time of exposure to iodine is critical. Longer treatment injures the cells and shorter exposure does not sterilize. Strips of skin 3 × 1 cm. are then cut and placed in physiological salt solution.

The recent development of numerous antibiotics and chemical bactericides has proved a boon to tissue culture and made possible the cultivation of many tissues hitherto rejected because of bacterial contamination. Naturally the use of these agents must be carefully controlled to avoid injury to the cells or imperceptible and misleading alterations in them. In this laboratory, we have obtained sterile cultures from human tonsillar tissue by washing the excised tissues in Tyrode solution containing penicillin and streptomycin from 50 to 100 units per cc. Penicillin in this concentration does not appreciably alter the pH of the solution, but streptomycin tends to acidify it. The pH should therefore be checked and corrected if necessary. The action of penicillin on various types of cells has been described by Herrell and Heilman (1943), by Cornman (1944a,b), and by Gey *et al.* (1944). Lewis and Richards (1945) tested the effect of DDT on cells in culture and various sulfonamide compounds have also been investigated by Jacoby, Medawar and Willmer (1941), by Jacoby (1945) and by Saunders and Haymaker (1945).

* Personal communication.

TUMORS

Several factors enter into the selection of *human tumors* suitable for cultivation. Necropsy material should be avoided. It is almost always nonviable and, unless obtained *immediately* after death, is contaminated. Tumors that have been heavily irradiated are also unsuitable since growth will be inhibited completely or reduced to a minimum. The fibrosis of primary tumors must also be considered. Careful dissection is necessary to obtain the interspersed soft nodules of active growth, Cameron and Chambers (1937). If these are dissected free of the connective tissue, vigorous growth may be obtained. Otherwise, the tumor cells will be mechanically restrained by the surrounding fibrous tissue. Metastatic tumors, with a minimal amount of fibrotic tissue, usually proliferate more rapidly in culture than do those of the original tumor.

From transplantable *laboratory tumors,* portions for culturing should be selected from the vascular area that will usually be found at the periphery of the tumor. The more central area may be necrotic and this of course should be avoided.

TISSUES FROM COLD-BLOODED ORGANISMS

Tissues from the adult larger forms are obtained aseptically by the usual sterile technique. For any amphibian material, the moisture of the skin exudate endangers sterility. The skin should be reflected away from the incision and held with artery clamps or weighted hooks. Care must be taken that no moisture from the skin seeps into the sterile cavity.

Small organisms such as tadpoles, squid and dogfish embryos, platy fish and fish fry, etc., may be sterilized by placing them in 1-10,000 sodium merthiolate solution or a saturated solution of sulfanilamide for 5 to 10 minutes before dissection. They are then rinsed in sterile Holtfreter solution, or sea water if indicated, and the extirpated tissues placed in Holtfreter containing penicillin and streptomycin 50 to 100 units per cc. Almost 100% sterile cultures may be obtained by this method. The same treatment is applicable to adults if skin is desired.

INVERTEBRATE TISSUES

The cultivation of invertebrate tissue is still lagging because of the attendant difficulty of obtaining it aseptically. We have obtained sterile tissues for cultivation from mollusks and clams by the method just described. Murray (1927), in her work with Planaria succeeded in sterilizing the tissues with ultraviolet radiation. Cary (1933), also used this method for *Ptychodera bahamensis* as well as repeated washings in sterile sea water with 10% hexylresorcinol. Frew (1928) cultivated larvae of the blow fly which he reared under aseptic conditions. Gavrilov and Cowey (1941) prepared mosquito tissue for culturing by treating the insect successively with methyl alcohol, Lugol solution, hyposulfite solution and finally by repeated washings in sterile Ringer solution. These manipulations were carried out on sterile filter paper before the removal of the desired organs which, in this instance, were the salivary glands. Upon removal, the glands were repeatedly washed in sterile Ringer and successful cultures were obtained. Carlson (1946) has succeeded in maintaining cultures of grasshopper neuroblasts "for long periods." His methods for obtaining sterility also involved repeated washings.

All of these methods are laborious and time consuming, but the recent development of antibiotics and their application to the preparation of this type of tissue will doubtless encourage more work in this field.

STORAGE OF TISSUES

It is preferable to cultivate tissues as soon as possible after their removal from the host, but in some cases it is necessary to store them for a period of time. There are individual differences as regards the ability of the cells to withstand storage. In general, embryonic cells are more adversely affected than those of the adult and wandering cells (lymphocytes and leucocytes) more adversely than connective tissue and epithelial cells. Wide variations also exist in the effect of storage on neoplastic cells.

The tissue should be cut in relatively small fragments, about 5 cm. in diameter, and placed in a tube containing physiological salt solution with 0.02% phenol red and 10% serum. A 2% solution of ash-free gelatine may be substituted for serum. The amount of fluid should be just sufficient to cover the tissue, leaving a relatively large air space above it. Store at 5°C.

Hanks (1948) reports on the viability of chick tissue after storage and also finds* that adult rabbit skin will remain in good condition for several weeks if the medium is renewed whenever the pH, as indicated by a color change in the phenol red, drops to 7.0.

METHODS FOR OBTAINING TISSUES FOR PURE CULTURES

There are three general methods for obtaining cells in pure culture. The first consists in successive culturing of a fragment of tissue rich in the desired cells in a selectively favorable medium, for example, fibroblasts and monocytes. The second consists in transplanting an isolated group of cells from a culture containing a mixed population and remounting it in a separate container. This requires care and technical skill. Pure kidney and thymus epithelium, and tumor cells have been obtained by this method. The third consists in using special procedures to dissect the tissue from the host in such a way that it is obtained pure at the source. When feasible this is probably the most satisfactory method and has been used by many investigators to obtain various types of epithelial cells and cartilage.

---

* Personal communication.

First Method

Fibroblasts are more easily obtained than any other type of cell. Carrel's "immortal" strain was started from the heart of a 10-day-old chick embryo and carried on by Ebeling (1922). Fibroblasts will grow from almost any other embryonic tissue. The early growth consists of macrophages, fibroblasts and, later on, muscle. If numerous transplants are made, especially in a medium rich in embryonic extract, the fibroblasts in the course of 3 or 4 weeks smother out the other types of cells, and a pure strain of fibroblasts is obtained.

Monocytes have been obtained from blood by Carrel and Ebeling (1922). To obtain a pure strain of monocytes, the blood is centrifuged before coagulation takes place and the fluid plasma carefully pipetted off —not decanted—taking care not to disturb the loose film of white blood cells, the buffy coat, lying on the upper surface of the red cells. To remove the buffy coat without disturbing it, it is necessary to induce clotting. This is done by gently adding two or three drops of embryo extract to the trace of plasma remaining on the buffy coat. Allow the tube to stand in an upright position at room temperature from 20 to 30 minutes, until the plasma with the adhering white cells forms a coagulum. If left too long, the clotting action will extend down to the red cells. This usually does not occur within an hour, although the period of time depends largely on whether the blood has been withdrawn in the first place in an expeditious way. When the plasma film has coagulated, flame the top of the tube thoroughly. By means of a sterile cataract knife loosen the clotted film of leucocytes from the wall of the tube and, gently tipping the tube, allow the film to slip into a watch crystal containing 2 or 3 cc. of Tyrode solution. The coagulum consists of two layers, a smooth upper layer of coagulated plasma to which the under layer of leucocytes adheres.

To remove any red cells which may come with the film, gently move it to and fro in the Tyrode with the point of the knife and keep washing with fresh Tyrode until all the loose blood is removed. Then slip a clean knife under the film and place it on a flat glass surface where it may be cut into fragments, 1- or 2-mm. square. Be careful not to permit the film to dry and, as soon as a fragment is cut, immerse it in Tyrode and treat it as any other tissue fragment.

If blood leucocytes are grown in Carrel flasks, which permit them to be washed and fed without disturbing them, most of the small polymorphonuclears and the small lymphocytes will disappear after about ten days, and the medium will become increasingly invaded with large mononuclear leucocytes. These as a rule do not form a tissue—adjacent cells do not fuse—but remain actively ameboid, sending out numerous pseudopodia.

Second Method

In this laboratory, we have cultivated pure cultures of kidney epithelium by cutting out growing sheets from selected areas. We have also obtained adult liver by the same method. Murray (1947) obtained pure cultures of thymus from rabbits one day to a year old. He reports better success by removing that portion of the culture containing the unwanted cells, and leaving pure tongues of epithelium *in situ,* to which he added fresh medium. Gey, Hanks and Barrett (1948) selected and removed small isolated colonies of tumor cells growing in roller tubes and from these obtained numerous strains of malignant cells in pure culture. Sanford, Earle and Likely (1948), obtained a pure culture of mouse sarcoma by picking up from a mixed population one tumor cell in a micro tube and planting the tube in a culture medium.

Third Method

Iris epithelium. A method for obtaining pure iris epithelium has been described by Fischer (1922a). He used chick embryos of 10- to 12-days' incubation. The writer prefers 8-day embryos. The eye is removed, a small incision made posteriorly, and the contents enucleated with an iridectomy forceps into a Tyrode bath. When the vitreous humor with the lens attached slips out through the incision, the circle of pigmented iris (either partial or complete) may be seen on the lens against a white background. The vitreous humor is discarded and the tiny line of black cut into two or three pieces. Some lens epithelium may grow with the pigmented iris and be indistinguishable from it since both tend to form a membrane. If this technique is carefully followed, however, no fibroblasts should be present. The *crystalline lens* of 5-day chick embryos has been cultivated for many months by Kirby (1927) and by Kirby, Estey and von Weiner (1933). This is a delicate technique and one which requires considerable experience and skill as the tissue is very fragile.

Thyroid epithelium has been grown by Ebeling (1925), by Carpenter (1942) and by ourselves from the glands of 18- to 21-day-old chick embryos.

Many other endocrine glands have been isolated and cultivated by numerous investigators, e.g., human and rat thyroid, and parathyroid by Stone, Owings and Gey (1934), parathyroid of newborn infants by Gaillard (1948), hypophysis of the rabbit by Kasahara (1935a,b), hypophysis of rats by Geiling and Lewis (1935), and by Anderson and Haymaker (1936), and ovaries of young mice by Francke (1948). Function was demonstrated in all of these cases but it is uncertain whether the cultures consisted of epithelial cells only.

Cartilage removed from the thick layer of the sclera of the eye of 15- to 18-day chick embryos was grown in pure culture as described by Fischer (1922b).

Chapter X

CHOICE OF METHODS FOR CULTURING

When the tissues and the ingredients of the medium have been prepared, the method of cultivation must be chosen. Cultures may be grown on coverslips, in flasks and in roller tubes, all of which have their variations.

The coverslip method as used by Carrel (1912) and by Lewis and Lewis (1911a,b) is the simplest and has signal advantages for the beginner. The fragment of tissue is placed in a drop of medium on a coverslip, covered with a depression slide, sealed and incubated at body temperature. The cells migrate and multiply and may be studied under the high powers of the microscope. This provides the basic requirement for acquisition of familiarity with the appearance of living, proliferating cells in tissue culture. The coverslip method is now chiefly used for studies of short duration. When a plasma clot is used, however, the method may be extended by repeated transfers of the cultures, in fact it was by this method that Carrel's strain of fibroblasts was maintained for 30 years. The frequent transfers to fresh medium containing embryo extract induce proliferation of the cells—the main objective of the coverslip method.

The flask and roller tube methods are better adapted to growth of larger amounts of tissue and to long-term cultivation with maintenance of functional activity. The tissue, embedded in a thin plasma clot and nourished by a supernatant fluid, may be left undisturbed for long periods, since the supernatant only is changed except when fragments are removed for cytological study or, at extended intervals, for subculturing.

The reduction of the frequency of transfers and limitation of growth-stimulating extracts reduces proliferation and enhances differentiation. This is especially evident in flask cultures where the plasma clot is thicker than in roller tubes. In flasks, the supernate usually remains quiet. In roller tubes, which are an evolution of the flask method, the tube is rotated so that the tissues are alternately exposed to nutrient and to air. Tubes are less expensive than flasks and are used effectively as a reservoir for tissues in continuous growth. Gey and Gey (1936) maintain various strains of malignant cells in this manner, obviating the necessity for large colonies of laboratory animals.

There are numerous modifications of these three methods (Fig. 21). Among the most satisfactory are the double coverslip method introduced by Maximow (1925) and that of Strangeways and Fell (1926a), Fell and Robison (1929), Fell (1939), who in studying developmental processes of embryonic organs, obtained excellent results by using watch crystals placed in Petri dishes containing moistened absorbent cotton. Strangeways and Fell (1926b) were also successful in the use of a simple test tube method. Hanks (1947) used test tubes in his studies of lepromata

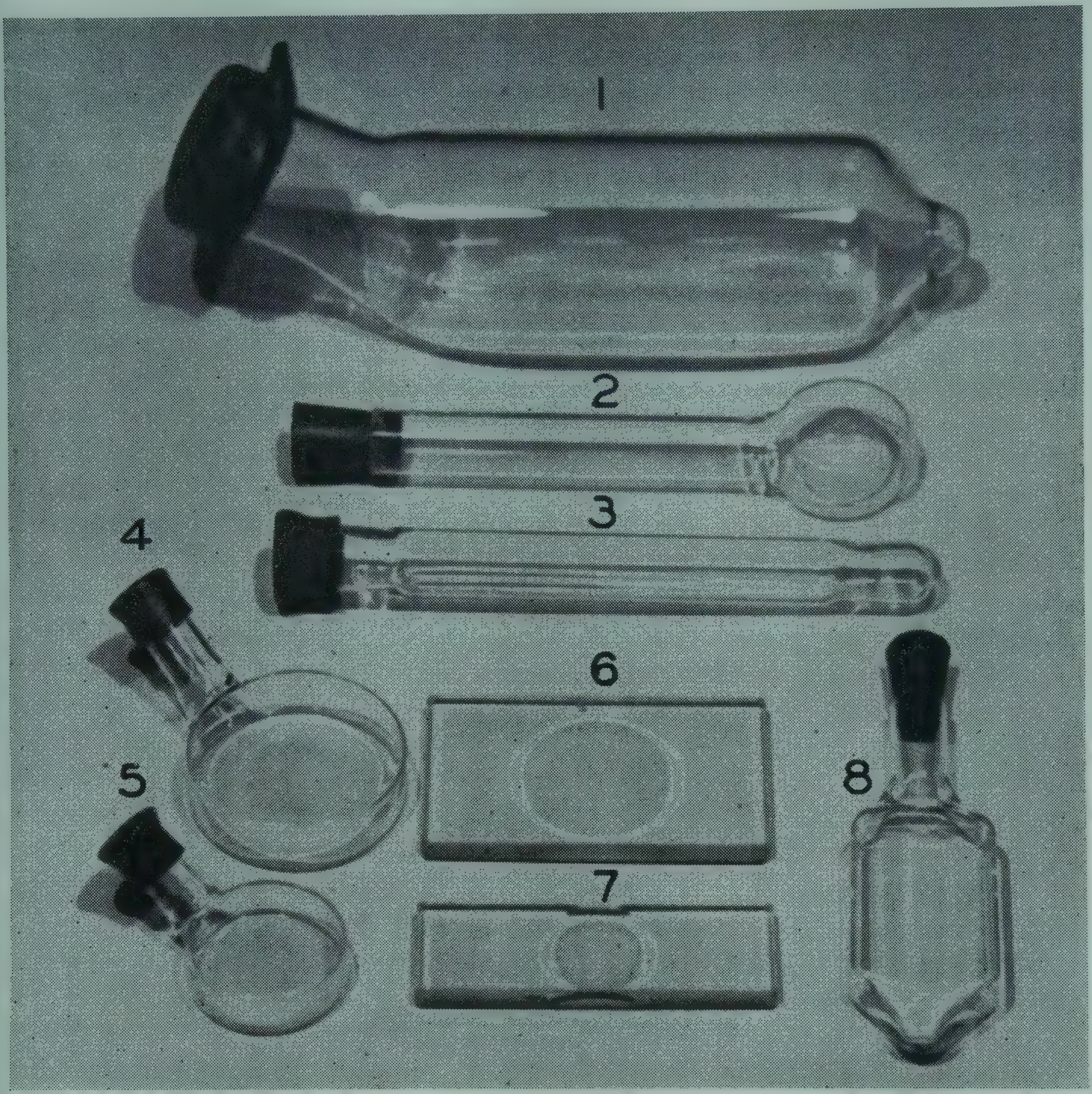


FIG. 21. Flasks and slides used as containers for cultures. 1, Grand's flask for study of the metabolic products of a large number of explants; 2, Porter's roller-flask; 3, Roller tube with flattened surfaces; 4 and 5, Carrel D_5 and D_3 flasks; 6, large depression slide for double coverslip method; 7, small depression slide for single coverslip method; 8, Earle's flask (used with perforated cellophane).

and Paff (1948) a modification of Fell's method for his studies of the effect of pH on the growth of bone. Sano and Smith (1930) use a glass or metal ring, covered top and bottom, with mica sheets as a container for their cultures. Willmer (1935) used a similar method by sealing removable coverslips over openings in the top and bottom of Carrel type flasks as did Dubin (1947) in his studies of malaria while Osgood and Brownlee (1936), Osgood (1938) cultivated bone marrow in fluid medium in 50 cc. vials.

The environment in which the cells are to live is equal in importance to the skill in preparing the cultures. The temperature of incubation and the composition and amount of the media are conditioning factors in successful maintenance of cells outside the body. The state of the cell is intimately affected by such factors as support for outgrowing cells as well as the chemical composition, hydrogen ion concentration, vapor tension and available oxygen of the clotted, fluid and gaseous phases of the medium.

As might be expected tissues grow most readily at the normal body temperature of the organism from which they were taken. Thus, for chick tissues the temperature for optimal proliferation is 38 to 38.5°C., for mammalian 37.4°C. and for cold-blooded forms, 18 to 22°C. Investigations have shown that temperatures below that for optimum growth inhibit multiplication of cells and tend to enhance differentiation.

CHAPTER XI

CULTURES ON COVERSLIPS

As has been mentioned, the primary purpose of the cultivation of tissues on coverslips was and still is that of fostering proliferation of the cells. Because of the small amount of medium used, frequent transplants into fresh medium are necessary for maintaining the tissues in active condition. This treatment stimulates multiplication of the cells which in turn suppresses differentiation. This chapter deals with the various methods of cultivating tissues on coverslips.

It will save time and mishaps if the operator learns to work in an orderly manner and keeps the culture table tidy as he works. Wrappings from sterile supplies, discarded stoppers, slides, pipettes, coverslips, etc., should each be placed in their proper receptacle as used. Cataract knives and other expensive fine instruments should never be flamed and should be cleansed immediately after use. If Bard Parker blades are used, flaming is permissible. As a rule, the preparation of the cultures immediately follows the removal of the tissues. The glassware and instruments which have been used for this purpose should be replaced with fresh ones. A routine for the various procedures should be established so that the necessary precautions will eventually become almost automatic and the operator will not be deflected from his experimental purpose by the need for constant consideration of each step of the technique. It facilitates the culture making to maintain a regular order for the placing of the pipettes containing the frequently used ingredients of the culture medium. For instance, the first tube could hold the chicken plasma pipette; the second, the embryo extract; the third, Tyrode solution; the fourth, serum; then two or three empty pipettes for different experimental substances; and in the last tube, a pipette of heavier caliber for Tyrode irrigation. Place tubes of the required medium in an upright position in a bowl of cracked ice taking care not to allow the solutions to splash the corks, since moisture on the stoppers may be a source of contamination. Small quantities of the media are stored in numerous separate tubes in order that fresh ones may be used frequently. In this way, any break in technique does not jeopardize the entire supply.

The granite cup containing the wax which is to be used for sealing the cultures should now be put over a low Bunsen flame or on an electric plate. A trace of vaseline added to the paraffin prevents it from chipping. Parawax, already prepared, is convenient.

SINGLE COVERSLIP METHOD

Fluid Medium

The fluid medium technique introduced by Harrison and developed by Lewis and Lewis (1911a,b) has several advantages. The method is comparatively simple, making elaborate equipment unnecessary. The cells grow as a thin sheet attached to the glass coverslip, which facilitates detailed study under high magnifications. The absence of fibrin in the medium is an advantage also for micromanipulative studies.

The method has its limitations, however. Cells require support for growth, which in this case they obtain by tenuous attachment to the coverslip. Slight vibrations detach them, whereupon they round up and die. Migration rather than extensive multiplication takes place and the life of the cells is usually limited to a few days. Precautions against infection need not be quite so meticulous as for cultures which are to be kept indefinitely. The simplicity of the technique makes possible the preparation of cultures in large numbers so that the few contaminations which may occur are not a serious matter. Certain precautions must be observed, however, to the extent that the tissue, media and any materials with which they come in direct contact, must be sterile, e.g., pipettes, coverslips, instruments, etc.

The medium may consist of a wide variety of solutions. Tyrode, Ringer, Locke, or other physiological salt solutions (formulas in Chapter V) with embryo extract and serum are frequently used media. Lewis (1916) has also used various dilutions of sea water mixed with muscle extracts, dextrose, etc., for marine forms. Three or four coverslips are placed in a row in front of the operator. They may be any convenient size that completely covers the hollow of the depression slides. The medium is drawn into a capillary pipette, a small drop of it is placed on each coverslip, and the drop spread with the back of a knife to a diameter of about 10 mm. The tissue fragment is placed in the center of the drop. The amount of medium in the drop should be just sufficient to form a film out of which the fragment protrudes somewhat, rather than a deep drop completely enclosing it. The importance of having the coverslips clean is here plainly manifest for, if the coverslips are greasy, the drop will not spread properly and the tissue will not grow.

To prepare a large number of cultures quickly a number of fragments of tissue in Tyrode may be drawn into a pipette and each piece deposited with a drop of Tyrode on successive coverslips directly from the pipette. The coverslips are then covered with a depression slide (with a small round concavity), which has been touched with a glass rod dipped in vaseline at each side of and a few mm. away from the round depression. The vaseline will temporarily fasten the coverslip to the slide so that it may be turned over for sealing with paraffin. A quick movement will prevent the drop from running. The wax should be sufficiently hot to be easily applied with a small camel's-hair brush. Apply the hot paraffin to the four edges of the coverslip as if painting them, and in such a way that the band of paraffin extends about 4 mm. onto the depression slide and the same distance onto the coverslip.

The purpose is to make the culture airtight to prevent evaporation of the medium and subsequent drying of the culture and also to prevent the entrance of bacteria. Therefore, precautions must be taken to see that the coverslip is entirely sealed onto the slide, as the slightest crack in the paraffin will ruin the culture. Label and place the culture in a rack in the incubator. Since the preparations will not be subcultured and are to be kept a few days only, vaseline alone is sometimes used for sealing. In this case the slides should be prepared before mounting the tissues. Use a pipette to surround the depression of the slide with a ring of heated vaseline and allow to cool before inverting over the coverslip.

Incubator racks are particularly useful in fluid medium technique. Usually better growth is obtained when the culture is kept in an inverted position, coverslip down. Since no fibrin is present, the cells must use the surface of the glass for support and the fragment of tissue should be in as close contact with the glass as possible and never allowed to hang free in the drop.

Because the cells are adherent to the coverslip, *transplantations* of cultures in fluid medium cannot be very successfully made. Their life may be prolonged, however, by opening the culture after 48 or 72 hours, carefully removing the fluid with a pipette or filter paper, and gently adding a drop of fresh medium. Des Ligneris (1936) has reported keeping tissues alive in fluid medium on coverslips up to 3 months. This he accomplished by careful control of the pH and by changing the fluid every day.

Both vertebrate and invertebrate tissue have been grown in fluid medium. The chief obstacle to the cultivation of invertebrates has been the difficulty of obtaining them in an aseptic condition. However, several investigators including ourselves have succeeded in sterilizing the tissues by various means discussed in Chapter IX.

Solid Medium

The culturing of tissues on a single coverslip in a clot composed of plasma and embryonic extract is usually called the Carrel technique. To prepare such cultures, place one or two coverslips (22 × 40 mm.) on the table with a sterile forceps. With practice, it is possible to make four to six cultures at one time.

For *chick tissue* place on a coverslip a drop of chicken plasma from a capillary pipette and add to it a drop of embryonic extract of the same size. Mix and spread the drops making the diameter of the spread drop about 10 to 12 mm. This is the culture medium. Then immediately, before coagulation begins, slip a knife under the piece of tissue to be cultivated (a needle may be used to help lift the tissue) and place it in (not *on*) the center of the medium. Cover with a depression slide touched with vaseline and put aside for two or three minutes until the medium coagulates. It may then be turned over and placed right side up to be sealed with paraffin before being placed in the incubator.

Mammalian tissues are done in the same manner. Good results with mammalian tissue, both normal and malignant, can be obtained by using chicken plasma, to provide the fibrin, mixed with serum and either chick or mammalian embryo extract. The procedure is that described for chick tissue with the exception that the medium must be mixed in a depression slide before it is transferred to a coverslip.

Place in a depression slide:

1 part chicken plasma
2 parts serum
1 part embryo extract

Mix well by drawing into a pipette two or three times. Then place two drops of the mixture, from a capillary pipette, on the center of the coverslip. Spread it with the back of a knife and place the tissue in the center of it. This must be done quickly before coagulation sets in. Cover with a depression slide and seal.

There are almost as many recommended media as there are investigators. Murray and Stout (1942a), for adult tissues, use a mixture composed of 2 parts chicken plasma, 5 parts human placental serum, 2 parts Simms serum ultrafiltrate, 1 part chick embryo extract. Gey routinely uses bovine extract and human placental serum in his medium, Earle prefers horse serum. All give good results. Some investigators use a completely homologous medium. To obtain mammalian plasma it is necessary to use heparin when drawing the blood (see Chapter VI). When heparinized plasma is used the coagulation time of the culture medium is increased. There are other disadvantages. Mammalian plasma contains less fibrin than that of birds and therefore provides a less firm coagulum and one that is more subject to the lytic action of growing cells. This is accentuated by the presence of heparin. Another disadvantage of mammalian plasma is its tendency toward increasing opacity, which in older cultures interferes with the observation of the cells in the cultures.

The fibrinogen, thrombin medium recommended by Porter and Hawn (1947) (see Chapter VIII), for flask cultures may also be used on coverslips. Porter stresses the importance of the concentration of the fibrinogen. If it is too high the cells are mechanically inhibited from migrating into the medium. The amount of thrombin used is not critical. Of several mixtures tried for cultures on coverslips we have found the best for chick tissues to be:

	Total concentration in medium	
1 part fibrinogen 0.8%	0.16%	
1 part thrombin 10 units/cc.	2 units/cc.	
2 parts Tyrode solution		use 2 drops
1 part chick embryo extract		

The substitution of serum for the Tyrode gave less satisfactory results. We found no entirely satisfactory mixture for mammalian tissues. If the fibrinogen concentration was low, the medium quickly liquefied and if it was increased, the cells did not migrate.

Tissues from *cold-blooded organisms* and from *insects* are cultured in similar manner although the medium must be somewhat altered. The physiological salt solution used throughout must be adapted to conform to the tonicity of the blood of the organism used. Sea water is substituted for Tyrode solution for marine forms such as clams and squid, Holtfreter solution for amphibia and fresh-water fish, Carlson's formula for insects (see Chapter V).

In general, we have found the most satisfactory medium for fish and amphibian tissues to be a mixture of

1 part fowl plasma	
1 part homologous serum	
1 part chick embryo extract (made in Holtfreter solution)	use 2 drops
1 part 4% ash free gelatine	

A gelatine solution made up in distilled water and autoclaved is more favorable than one made in Holtfreter solution and sterilized in a water bath. Presumably, the autoclaving breaks down some fraction of the protein which is utilized by the cells. If homologous serum is unobtainable, most cold-blooded tissues, especially those from amphibia, grow equally well in a medium composed of equal parts of fowl plasma, gelatine solution and tissue extract. In recent experimental work, we have found that extracts of whole tadpoles or adult frog muscle (see Chapter VII) are even better than that made from chicks. The cultures are *not* placed in the incubator but kept in a cool dark place at from 18 to 22°C.

Among the investigators who have cultivated tissues from cold-blooded animals are Uhlenhuth (1914, 1916), Holmes (1914), Dederer (1921), Gatenby (1932), and Preston (1948).

The medium used by Gavrilov and Cowey (1941) for mosquito tissue consisted of chicken plasma, chick embryo extract and filtered extract of mosquito larvae. It is well known that chick and mammalian extracts are inactivated by filtration, so it is doubtful if the mosquito extract acted as other than a diluent. However, these investigators report they obtained good growth.

Carlson's (1946) medium for tissues from grasshopper embryos consisted of Carlson's solution with the addition of a "small amount" of grasshopper egg yolk. In this medium he maintained his cultures "for long periods."

SUBCULTURES

After 24 hours of incubation, embryonic tissues will show a circle of new growth which, after 48 hours, will be sufficiently large to warrant subculturing. Most cultures will survive for about a week without subculturing, but if it is desired to keep the cells in an active condition, it is better to transfer them to a fresh medium every 2 or 3 days when using the coverslip method. This is done in the following way:

Carefully examine the culture under the microscope for bacterial infection. If contaminated, the culture must be discarded as transplantation spreads the infection. Common contaminants from faulty technique are staphylococci (*aureus* and *albus*) which grow in smooth, round colonies; *Sarcina,* identified by their peculiar arrangement in cubes of 8 or more; and *B. subtilus,* a common air contaminant, which spreads very rapidly over the medium and often causes marked liquefaction. Spores of yeasts and molds are also usually air-borne or may derive from the skin of amphibia. The long branching mycelium of the latter grow very rapidly and soon cover the entire culture medium. The spores are very resistant and, if this type of contamination is found, the unopened culture should be autoclaved.

The next step is facilitated by the use of the bakelite block on the table board. The paraffin, while still warm from being in the incubator, is loosened with the blade of a discarded scalpel or cataract knife and the coverslip containing the culture turned face up on the bakelite square. Care must be taken to remove all the paraffin so that a smooth surface remains. Otherwise pressure of the knife may break the coverslip. See that no loose paraffin drops on the coverslip, or infection will result.

Hold the edge of the upturned coverslip firm with the thumb and forefinger of the left hand, if 22 × 40 coverslips are used. Smaller ones are held with the tip of a sterile needle in a holder. The culture is cut with a cataract knife, held, not like a pencil, but parallel to the coverslip. Remove the old plasma surrounding the culture by cutting *through the edge* of the new growth, *not* outside of it (Fig. 22). To obtain a good subculture, it is necessary to make a sharp clean cut, leaving no rough tag ends of the old clot. If old plasma remains, the transfers will grow slowly and unevenly. If the fragment is to be divided, cut it through the center. Remove the pieces from the old clot to a Tyrode bath for two or three minutes; if they have curled up during manipulation, see that they float out flat again before mounting them in new medium on a fresh coverslip.

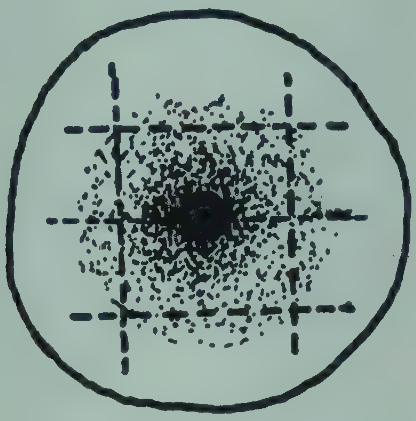


FIG. 22. Diagram to show method of cutting cultures for transplantation.

While the plasma-embryo extract mixture was a distinct advance over the fluid medium, the method of repeated transplantation is arduous, demanding skill and patience. The small amount of medium and of available oxygen makes frequent transfer necessary, which entails disturbance of the cells and retardation of growth during the period of readjustment to the new environment. Moreover, frequent transplanting stimulates growth of fibroblasts, and inhibits organization and functional activity. These disadvantages are reduced by the use of double coverslips.

DOUBLE COVERSLIP METHOD

This method, introduced by Maximow (1925), has the same adaptation to high power microscopic observation as the single coverslip, with the additional advantage of labor saving and infrequent disturbance of the cells. The tissue may grow undisturbed for periods varying from 10 days to a month, depending on the type of tissue and its rate of growth. When this method is used the fibroblasts are not over stimulated by frequent transfers, multiplication of cells is somewhat reduced and differentiation is therefore possible. We find the double coverslip method very satisfactory and have used it extensively. Murray and Stout (1947b) use it in cultivation of tumors for diagnostic purposes, Pomerat and Anigstein (1944) for his work with immune serum, Goldfeder (1940), and Goldfeder and Cameron (1948) for radiation of tissue cultures.

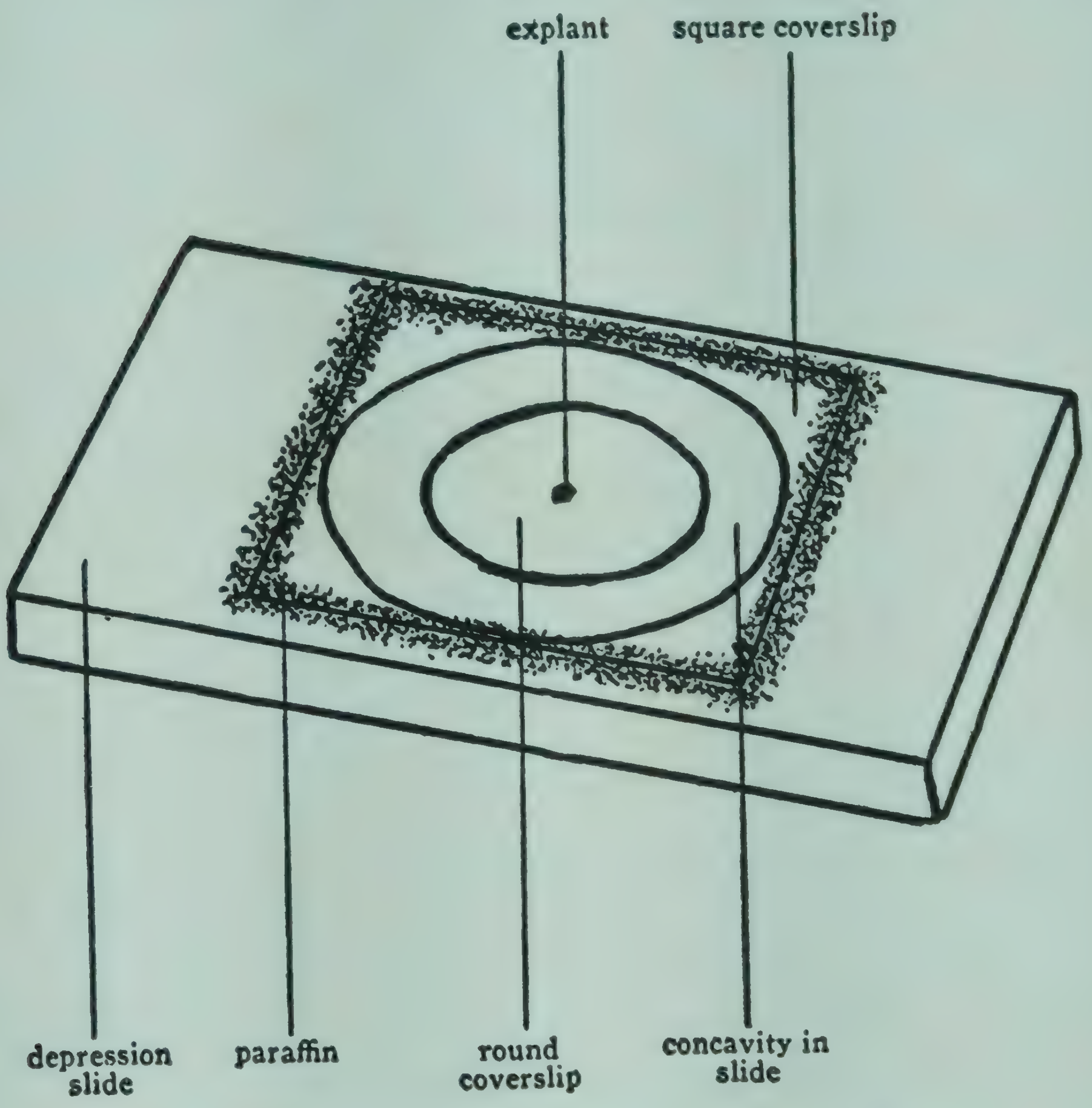


Fig. 23. Diagram of culture on double coverslip.

The method may be used for any type of tissue and is particularly well adapted for the cultivation of mammalian tissues, especially tumors, which exhibit a more or less marked tendency to liquefaction of the medium. We have also found it useful for cultivation of tissues from cold-blooded organisms. Tissues from any source are cut in a suitable physiological salt solution and mounted in the following manner:

Place two or three square coverslips (preferably of mica), 40 × 40 mm., on the table, and onto the center of each one drop a very small droplet of Ringer or Tyrode solution. On this drop of saline, by means of sterile forceps, place a No. 1 round coverslip 22 mm. in diameter. Suction will cause adherence of the coverslips. Then proceed as described for single coverslip cultures placing first the medium, and then the tissue on the coverslip. Cover with a large depression slide (45 × 75 mm. with 38-mm. round concavity) and seal the mica coverslip onto it with paraffin (Fig. 23). Place in the incubator in an inverted position in the rack to provide a lying drop instead of a hanging drop. The serum which accumulates on the surface of the clot readily drops off a hanging drop and gathers at the edges of the round concavity, endangers sterility, and sometimes leaves such a thin layer of medium over the surface of the clot that the cells are adversely affected.

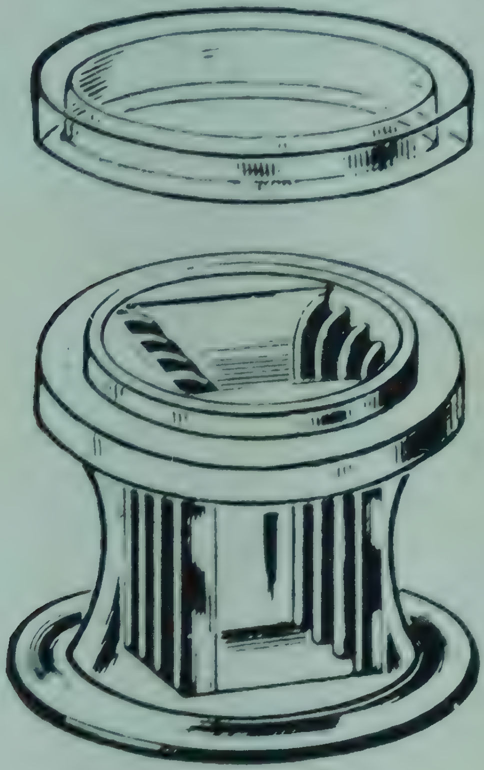


FIG. 24. Columbia staining dish.

As the tissue grows, liquefaction begins and increases in proportion to the activity of the culture. This is especially marked in cultures of neoplasms. The culture should be washed and fed every 2 or 3 days. In this procedure the advantage of the mica coverslip will be seen. The wax is removed from the mica square. Using the left hand, the farther edge of the mica is held in place on the slide by the tip of the second or third finger, the thumb lifts the near edge of the mica and light pressure of the first finger against the central area bends the mica and breaks the suction so that the round coverslip may easily be removed by sterile forceps. The coverslip containing the tissue is bathed in Tyrode solution in a Columbia staining dish (Fig. 24) for about 10 minutes. In this way the old liquefied medium is washed away as well as the waste products of cellular growth present in the remaining clot.

The circle is then lifted from the Tyrode by fresh sterile forceps and placed on a clean, dry, square mica coverslip. If the coverslip is removed from the Tyrode bath slowly, allowing time for the fluid to run off, only sufficient Tyrode will remain on the circular coverslip to cause it to adhere to the square. If removed quickly too much liquid may be carried over, in which case the excess must be removed with a strip of sterile filter paper. Otherwise the round coverslip will slide to the edge of the mica, endangering sterility. A drop of fresh medium is then added to the remaining portion of the original clot. If much liquefaction is present the added medium may consist of a drop of the original plasma medium; if little or no liquefaction has occurred the plasma may be omitted and a drop of a mixture of serum, Tyrode and extract added. This procedure of washing and feeding the culture may be kept up for varying lengths of time, depending on the rate of growth of the tissue, the condition of the cells and the amount of liquefaction of the medium.

Embryonic tissues may need transfer every 2 or 3 weeks, adult tissues less frequently. When subcultures are to be made, the tissue is cut, washed, and transplanted as described for tissues on single coverslips. It is advisable to remove the circular coverslip to a fresh sterile square before cutting the tissue as the least trace of paraffin on the mica makes the cutting surface uneven. The cut fragment of tissue may be remounted in any desired way.

Cultures on coverslips may be fixed and stained *in toto* when further cytological study or permanent records are desired. The methods will be found in Chapter XVII.

CHAPTER XII

CULTURES IN FLASKS

As has been pointed out, the coverslip methods of culturing are favorable for inducing proliferation of the cells while flasks permit differentiation. Carrel (1923), experimenting with methods for maintenance of cultures for longer periods without transfer, developed several types of culture flasks. In these ample nutriment and oxygen are provided, growth of the numerous tissue fragments is more nearly uniform under identical conditions, and the interval between the disturbance of transfers is greatly lengthened. Other investigators have designed various flasks to suit their purposes (Fig. 21). One by Grand is for the cultivation of large amounts of tissue for studies of the products of cell metabolism in the supernate, while that of Evans and Earle (1947) was evolved in the search for a substitute for the fibrin clot. The chief disadvantage in the use of any flask lies in the difficulty of high power observation of the cells, although this has been partially overcome by improved methods of manufacture, by the production of microflasks of optically clear glass and by the use of the inverted microscope, see Chap. XVIII. Permanent records by means of fixation and staining can be obtained only by sacrificing the flask or by removing the tissue and remounting it on coverslips. The basic technique for cultivation of tissues in flasks by this method will be described in this chapter.

MATERIALS AND METHOD OF PREPARATION

Carrel flasks
Rubber stoppers to fit the flasks
Pipettes—graduated in tenths of a cc.
Platinum spatula
Platinum hypodermic needle

Of the several types of flasks designed by Carrel, the most generally serviceable is the circular D_5, which is 5 cm. in diameter and about 1 cm. deep. It has a slanting arm, 3 cm. long and 1 cm. in diameter. The D_3 is of the same design but with a diameter of 3 cm.

Prepare the fragments of tissue to be cultivated in Tyrode as previously described. Place a 5 cm. Carrel flask on the table with the neck toward the right of the operator. Remove the cotton plug and introduce 0.5 cc. of the plasma by means of a graduated pipette. It is important to tilt the flask immediately, in such a way as to cause the plasma to cover the entire floor of the flask before any clotting begins. If this precaution is neglected, the clot may be uneven and will probably become detached from the flask before the expiration of the experiment. Add 1.3 cc. of Tyrode solution and 0.2 cc. of embryo extract to the still fluid plasma and move the flask gently to mix the medium. Using the spatula, introduce the fragments of tissue and orient them in the medium before coagulation sets in. Five or six fragments may be grown in a flask 5 cm. in diameter.

Flame the opening of the flask and seal with a hard rubber stopper. "No-air" red rubber stoppers are excellent. They have a solid rubber core as well as a flange which covers the entire edge of the mouth of the flask. The stopper must fit tightly to prevent loss of CO_2 with the subsequent development of excess alkalinity. Do not move the flask to the incubator until the clot is firmly coagulated. This usually takes about 5 minutes and can be tested by gently tilting the flask.

After the flask has been in the incubator an hour or two, add 1 cc. of nutrient fluid medium. The nature of the experiment will determine the constitution of the supernatant fluid. In any case it has been found that the growth promoting and protective factors of tissue extracts and sera are nonspecific and may be used for any type of tissue if isotonic with the blood of the organism from which the tissue was removed. For routine cultures the supernate consists of

40% Tyrode solution
40% serum (human, horse or rat)
20% embryo extract (chick or mammalian)
0.01% phenol red

The presence of phenol red in the supernatant fluid in this concentration has no adverse effect on the cells and makes it possible to check the hydrogen ion concentration at a glance. As the tissue grows, CO_2 will normally increase and the pH of the medium become correspondingly more acid as indicated by a gradual change from the rose color of pH 7.6 toward the yellow of pH 7.0. The color change is helpful in determining when the fluid or gaseous medium should be changed as most tissues grow best at about 7.6. A striking or sudden shift to the acid side would suggest a possible contamination. If maintenance or differentiation rather than proliferation are desired, the amount of Tyrode solution should be increased and the embryo extract reduced or eliminated. The serum must be retained for its protective action in preserving the osmotic balance between the cells and the medium.

For the smaller D_3 flasks, the amount of medium is reduced proportionately to 0.3 cc. of plasma, 0.6 cc. of Tyrode, and 0.1 cc. of embryo extract with 0.6 cc. of supernatant nutrient. Each investigator has his own preferences in regard to the exact constitution of his culture medium. Those mentioned above are used in this laboratory.

The constitution of the gaseous medium must also be considered. For routine cultures we rely on the oxygen of the air and introduce special gas mixtures for specific purposes only. If the medium is unduly alkaline it may be adjusted with the CO_2 of expired alveolar air. A simple method for doing this is to allow one's expired breath to pass into the flask through a pipette of heavy caliber containing a loose cotton plug. Tanks of CO_2 in air (5% is convenient) are obtainable commercially or separate tanks of oxygen, carbon dioxide and nitrogen may be used to make any desired mixture in the laboratory. Gas from a tank should be filtered by some means before introducing it into the culture flask. This may be done by attaching one end of a length of rubber tubing to the valve of the tank and the other end to a gas washing flask containing a fritted gas filter (Corning). A length of sterile rubber tubing is attached to the other arm of the filter flask through which will pass the filtered gas to an attached sterile pipette introduced into the flask. A Berkefeld filter or a cotton filled glass bulb may also be used.

The supernatant fluid medium should be removed every 2 or 3 days, the culture bathed with Tyrode for 10 or 15 minutes, and fresh nutrient fluid added. For withdrawing the fluid, a flamed platinum hypodermic needle attached by rubber tubing to a suction pump is very convenient, but a pipette may be used if suction is not available. Occasionally, the clot may become partially liquefied. It is then necessary to add a few drops of plasma and extract to assure adequate cell support. The exact amount to be added will depend on the degree of liquefaction.

The length of time that cultures may remain in a clot without deterioration varies considerably. Rapidly growing embryonic tissues, such as chick fibroblasts, usually need to be subcultured every 10 to 14 days. The more slowly growing adult tissues may be left undisturbed much longer. Blood leucocytes, which wander throughout the entire medium, remain in excellent condition for 3 or 4 weeks. The operator, after examination of each fragment, will soon be able to judge the treatment required according to the condition of the cells. When a dilute nutrient is used, less frequent transfer is needed. If a culture is kept for several months in the same flask the plasma clot gradually becomes opaque. This condition, while making microscopic examination of the cells difficult, does not appear to affect them adversely.

Since the tissue grows on the floor of the flask, unless an inverted microscope is used (Fig. 45), the flask must be inverted for microscopic examination and special care must be taken to prevent the fluid from touching the stopper. In the inverted position the flask rests on the rubber stopper at one side and on the edge of the flask on the other and tends to be unsteady. A special holder may be devised such as that used by Earle (1943). In this laboratory, we place our inverted flasks on an ordinary glass slide and press a small piece of plasticine against the edge of the flask resting on the slide. This holds it firm yet facilitates free movement of slide and flask across the stage of the microscope.

For *subculturing,* the clot should be removed whole if possible and, after a little practice this usually can be done. Removal of the clot intact is facilitated by the addition of a "patch" of plasma and extract the day before the transfer is to be made and by placing the flask in an inverted position for several hours or overnight before the transfer. By this means the fluid drains out of the plasma coagulum and a tougher, less friable clot is obtained. The flask is held neck up, the blade of the spatula inserted and carefully worked between the glass and the clot. When the clot is partly loosened, a small amount of Tyrode introduced between it and glass may help to detach it completely. Flame the neck and let it cool; then pour the contents of the flask onto the sterile cover of a Petri dish. We have found this better than a flat glass plate as the elevation of the Petri dish facilitates cutting. If unbroken, the clot is easily spread out as a sheet. Cut the tissues with a cataract knife, taking care to discard as much as possible of the old plasma clot, and transfer the tissue to a depression slide containing Tyrode solution. Reculture as desired.

The flask method is excellent for large amounts of tissue or when it is desired to grow tissues undisturbed for a length of time; but the fact that the containers must be handled many times in the course of an experiment makes the utmost precautions imperative to avoid contamination. Flame the necks of the flasks each time they are opened and use fresh stoppers. When examining a culture, see that the fluid medium never flows into the neck of the flask, nor touches the stopper.

The use of a clot composed of fibrinogen and thrombin for cultures in flasks has been reported by Porter and Hawn (1947) and Favata (1947). The solutions of these substances are made up as described in Chapter IX. A flask 3 cm. in diameter is prepared as follows:

		Total concentration in the medium
Fibrinogen 0.5% or 0.25%	1 part	0.16% or 0.08%
Nutrient	2 parts	
5 parts Tyrode solution		
3 parts placental cord serum		
2 parts embryo extract		

Of the fibrinogen and nutrient mixture 1 cc. is introduced into the flask and 1 drop of thrombin solution containing 0.5 to 1 unit is added. This is mixed and the fragments of tissue immediately placed in position before coagulation occurs, which it does in about 1 minute. At room temperature, if the mixture is about pH 7.6, the clot will be transparent. Ice-cooled nutrient and fibrinogen and a pH lower than 7.5 during clot formation will result in a certain degree of opacity. After a few hours incubation at 38°C., the clot will undergo syneresis and exude about one half of its volume. The cultures are fed twice weekly with 1 cc. of nutrient fluid. No difficulty is encountered in subsequent transfers of the cultures.

Evans and Earle (1947) introduced the use of *perforated cellophane* as support for cells growing in flasks. Either the round Carrel D_5 type or an elongated one of Earle's design may be used. The perforated cellophane (du Pont) is cleaned by passing it through acetone, alcohol, ether and redistilled water. It is then cut to fit the floor of the flask and placed in it before autoclaving. For preparation of the culture, 1 cc. of nutrient is introduced into the flask and a few moments allowed for the softening of the cellophane. The tissue is then placed in position between the floor of the flask and the cellophane. Earle routinely replaces the air in the flask with a mixture of 5% CO_2 in air. Under cellophane fresh tissue grows relatively slowly at first, but after a few days grows rapidly, for the most part in thin sheets attached to the cellophane. Two thirds of the fluid only is changed three times weekly. When the cells have covered a large proportion of the cellophane, they are scraped from it with a platinum spatula and the suspension of cells transferred to fresh flasks. This is an excellent method for the cultivation of thin sheets of cells. The type of growth does not foster organization nor physiological function dependent on it.

Erlenmeyer flasks are sometimes used for the cultivation of tissues in fluid medium. Parker (1936) maintained large quantities of adult tissue in serum and Tyrode with daily changes of the gaseous phase of the medium. Obviously no cytological study of the living cells is possible by this method. At the termination of his experiment, Parker made histological sections and reported that the tissues were in good condition and functionally active after a year in one flask.

In the field of *virology,* the tissue culture technique has made an outstanding contribution to medical science. It is well known that most viruses multiply only in living tissues and preferably in those actively growing. Tissue culture provides a simple means for the propagation of viruses and rickettsias with large yields of material of high titer for investigational purposes and for the preparation of vaccines. The reader is referred to Ender's (1948) comprehensive review of this subject.

In this laboratory we have demonstrated inclusion bodies in inoculated cells on double coverslips: Broadhurst, Cameron and Taylor (1938), Broadhurst, Cameron and Saurino (1938), Grand (1944, 1949). Morgan and Wiseman (1946) propagated psittacosis virus in roller tubes.

Flasks, however, are most frequently used for virus cultivation. Grand uses a large flat container with an oblique neck and a reservoir at the other end for collecting the supernatant fluid. It is capable of accommodating about fifty fragments of tissue. The tissue is embedded in the flasks in the usual thin plasma clot and the inoculum added to the supernatant fluid medium. The fluid is removed after a few days and replaced with the normal mixture of Tyrode, serum and embryo extract, 5-3-2. This fluid is collected and used for titration or for reinoculation.

Most investigators make use of Erlenmeyer flasks with fluid medium although roller tubes and Zinsser's agar slants have also been used. A long list of viruses and rickettsias have been cultivated in tissue cultures including vaccinia by Steinhardt, Israeli and Lambert (1913), Parker and Nye (1925), and Li and Rivers (1930). Psittacosis has been grown by Bland and Conti (1935) and by Morgan and Wiseman (1946), influenza by Francis and Magill (1935), poliomyelitis by Sabin and Olitsky (1936), yellow fever by Theiler and Smith (1937), and rabies by Webster and Clow (1937). The constituents of the medium, the temperature of incubation and the oxygen requirements vary for the different viruses.

Simms and Sanders (1942) developed a serum ultrafiltrate from ox blood which they recommend for the propagation of numerous viruses. Parker and Hollender (1945a) report the successful use of this ultrafiltrate as a medium for mouse encephalomyelitis and for rabies virus (1945b). Zinsser and Schoenbach (1937) have related the oxygen consumption to the potency of the virus at different periods of incubation, and many investigators report that temperatures lower than those most favorable for cell growth increase the yield of the virus.

Many valuable findings have been reported concerning the specificity of some viruses for certain cells. Carrel (1926) found that the Rous chicken sarcoma virus is associated with the wandering cells (leucocytes and macrophages) and that it would not multiply if fibroblasts alone were present. Findlay (1928) showed that fowl pox virus to which rats and mice are not susceptible will not grow in cultures with a substrate of rodent tissues. Similarly, Maitland and Maitland (1931) and Hecke (1931), studying foot and mouth disease reported that the presence of epidermal tissues is necessary for propagation of the virus. Fowls are known to be immune to this disease and the virus will not grow in chick tissues.

The changes in pathogenicity of viruses in tissue culture may also be studied. Two types of vaccines have been obtained, one in which the virus becomes weakened by successive passages, Rivers and Ward (1935), Theiler and Smith (1937); and the other by subjection to ultraviolet radiation, Hades, Webster and Lavin (1940).

Chapter XIII

CULTURES IN ROLLER TUBES

"Esmarch rolls" were used in bacteriology as long ago as 1886, but roller tube tissue cultures are essentially an outcome of the flask method. They were initiated by Gey (1933) for "massive tissue culture" and further developed by Gey and Gey (1936) and by Lewis (1935b). Ordinary Pyrex test tubes are used as the culture container which is mechanically rotated to expose the tissues alternately to a fluid nutrient and to oxygen.

Materials Required

Pyrex test tubes 150 × 50 mm. without lip
Solid rubber stoppers to fit
Pipettes graduated in tenths of a cc.
Pipettes 180 mm. in length with bent tips

PREPARATION OF CULTURE

Nutrient Solution

This may consist of	*or*
50% Tyrode solution	40% Tyrode solution
30% serum (human cord, horse or rat)	40% serum
20% embryo extract (chick or mammalian)	20% embryo extract
0.01% phenol red	0.01% phenol red

Prepare the tissue for cultivation and place five or six fragments in 3 drops of the nutrient solution. Introduce 2 drops of fowl plasma into a tube, rotating the tube and spreading the plasma with the back of a bent tip pipette. The plasma should moisten the inner surface including the bottom of the tube to within 2 inches of the top of the tube. Then draw the fragments of tissue and the 3 drops of nutrient into another bent tip pipette and place them in a vertical row along one side of the inner surface. The first fragment should be about 20 mm. from the bottom and the others spaced 15 mm. or more apart. Rotate the tube to mix the nutrient and plasma, then hold the tube upright for a few moments and withdraw any excess medium which may accumulate at the bottom of the tube. Place the tube in a horizontal position, preferably on a rack such as is shown in Figure 13, until the medium is coagulated. If a *very* thin film of medium is desired, place the tube during coagulation so that the tissues are on the upper surface and the medium will drain away. Otherwise the tube is so placed that the tissues are on the lower side. As with all cultures in plasma medium, it is necessary to work quickly to orient the tissues before coagulation occurs. Some workers use a small amount of heparin in the plasma to retard coagulation. We have not found it necessary to do so if the tissues are previously prepared and the procedures organized for efficient work. In warm weather it is helpful to chill the tubes before preparing the cultures and the media should always be kept in chopped ice during operation unless a culture room refrigerator is immediately at hand.

After complete coagulation add 0.7 to 1 cc. of nutrient as the supernatant fluid medium. If a special gaseous medium is desired, it is introduced in the manner described in the previous chapter for flask cultures. The tubes are then placed in the incubator in a revolving drum (Fig. 12) which is set at an angle of 5°, just sufficient to prevent the fluid from touching the stoppers. The drum is rotated 6 to 8 times an hour. The fluid and gas phases of the medium are changed twice weekly. Occasionally, particularly if neoplastic tissues are being cultured, liquefaction of the plasma film may occur. Whenever necessary a drop or two of plasma may be added.

For microscopic examination of tube cultures, a holder for the tubes is necessary. Gey uses one made of two flat pieces of brass about 8-inches long fastened together at the ends in such a way as to form a groove to support the sides of the tube, leaving an unobstructed space between the supports for observation. One end is weighted to keep the holder in place on the stage of the microscope and to prevent tipping. The microscope is tipped sideways by placing a wedge under one side to prevent the fluid from touching the cork. The holder we use is a modification of Gey's and has a slightly raised support for the upper end of the tube to prevent the fluid touching the stopper. In this case tilting of the microscope is not necessary (Fig. 14).

The alternate exposure of the tissues to oxygen and nutrient makes frequent subculturing unnecessary. Most tissues, especially those of adult origin, need not be transferred oftener than every 3 weeks. One fragment may be removed at any time for high power observation without disturbing the others. The method of removal is the same whether one or all of the fragments are to be transferred.

For *subculturing,* the original tissue and the surrounding area of new growth are "ringed" with a bent pipette. In other words, the film of plasma around the cells is broken. The film containing the cells is then gently loosened from the glass by pushing it from the periphery toward the central fragment. When the tissue is loosened, it is drawn into the pipette with a little of the supernate and deposited on a slide for trimming. If all the fragments are to be removed it is usually more convenient to start at the top and work down, but any fragment desired may be extirpated. The tissue is cut as described in Chapter XI and may be recultured in any manner.

Sometimes the medium is removed by digestion with trypsin solution as suggested by Vogelaar and Erlichman (1934, 1939). We prepare a 2% trypsin solution in Ringer or glucosol which is centrifuged and filtered by pressure through a Seitz filter. This solution may be stored in the refrigerator for several weeks. Immediately before use, a 0.5% solution is made by dilution with Tyrode solution. The filtration reduces the activity somewhat, but sufficient active principle remains for digestion of the thin film within a few minutes. When the clot is liquefied, the tissue fragments may be drawn into a pipette. It is necessary to wash them thoroughly so that no trypsin is carried over to digest the clot of the fresh culture.

In some experiments, the plasma film is omitted when preparing the tube cultures. The tissue is then placed directly on the surface of the glass where it is allowed to dry sufficiently to induce adherence to the glass before adding the supernatant nutrient fluid. This method is used by White (1946) in his studies on synthetic media. Obviously it is not to be recommended except for specific experimental purposes since the desiccated cells are destroyed.

A clot composed of fibrinogen and thrombin is used by Porter and Hawn (1947) primarily in the roller flask (Fig. 21) (see Chapter XII). Other modifications have been developed to mitigate the difficulty of a lack of a uniformly flat field for high power observation. Gey and Gey (1936) use a specially constructed hexagonal tube with flat surfaces of optically clear glass and a restricted neck to prevent overflow of the fluid and possible contamination. We also use a thin walled rectangular tube for special purposes, and some investigators use a bottle with flat sides. Shaw, Kingsland and Brues (1940) describe such a roller bottle. In one side of the bottle a hole is drilled and the opening covered by cementing a coverslip on which the tissue is to be cultured over the aperture. The rotating drum must of course be adapted to hold the type of container used.

If it is planned to remove the tissues without disturbing them for high power observation or for fixation and staining at the end of an experiment, coverslips on which to grow the tissue may be inserted into the roller tube or roller flask and fastened to the wall by a drop of plasma and extract. We have also used strips or squares of both plain and perforated cellophane for this purpose. Generally speaking, in our experience the cells grow better on the glass coverslips than on the cellophane.

Any type of tissue, chick or mammalian, embryonic or adult may be grown in roller tubes. Recently we have cultivated various tissues from amphibians by this method. The tissue fragments are fastened to the tube surface by fowl plasma in the usual manner with a supernatant nutrient consisting either of

40% Holtfreter solution
20% serum (horse serum diluted with 25% water, or frog serum)
20% 4% ash-free gelatine in water, autoclaved
20% chick embryo extract in Holtfreter

or

50% Holtfreter solution
30% gelatine solution
20% embryo extract

The preparation of the medium for amphibian tissues has been discussed in Chapter XI. For the amphibian cultures the temperature of the incubator housing the drum is maintained at 18 to 20°C. and the supernatant nutrient changed every 5 to 8 days. Excellent growth of epithelium and chromatophores was obtained from embryonic and adult frog skin (Figs. 34, 35). Connective tissue, muscle and epithelium from the pituitary gland also grew luxuriantly.

Roller tubes are inexpensive, easily obtainable and roller tube cultures require less attention than flask or slide cultures. They have proved valuable for long-term cultivation of tissues and are used by Gey, Hanks and Barrett (1948) and by others for maintenance of various strains of cells including those of tumors. The method is also well adapted to the study of the products of cellular metabolism and White (1946) uses this method for the determination of the nutritional requirements of cells. Its use for detailed cytological study is limited.

CHAPTER XIV

WHAT ONE SEES IN TISSUE CULTURES

The beginner is frequently confused as to the identity of the cells appearing in his cultures. Originally, attention was centered almost entirely on the cells growing out from the explanted fragment but tissue culture in its wider and more recent aspects deals also with developmental processes, organization and function. For this type of study the explant as well as the outgrowth should be given consideration.

THE EXPLANT

There has been a tendency to discount the value of the explants, *per se.* If the implanted fragment is too large, it becomes increasingly necrotic with the age of the culture and the necrotic regions exert a deteriorating effect on the culture as a whole. This difficulty is obviated provided the fragment is not over 1 to 2 mm. in diameter and is not unduly injured in cutting. With such precautions the explant itself furnishes material for fruitful study. After about 24 hours incubation, such explanted fragments spread out and become thinner, facilitating observation of the cells within the explant. While the explant is spreading an outwandering of cells occurs along its margin and eventually many explanted fragments become almost indistinguishable from the sheets of cells growing from them. In both central and marginal areas progressive development will take place such as the formation of Hassel's bodies in cultures of thymus, the deposition of colloid in thyroid gland follicles, and development of fat droplets in cells of both normal and malignant mammary gland.

Figure 25a shows part of an explant of chorio-allantoic membrane of a 9-day chick embryo with blood vessels developing and extending with the new growth. Figure 25b shows a growing pancreatic duct extending from the explant (not shown in figure). The lumen is being developed by the formation of cyst-like cavities which eventually merge and are filled with fluid, presumably, secreted.

In work done in this laboratory on the secretory processes of kidney tubules of meso- and metanephros from various organisms, we have paid more attention to the already organized tubules within the spread out explants than to the cellular outgrowth, Figure 26. The tubules, especially in the presence of a sulfonphthalein indicator such as phenol red, are sharply delineated as the indicator is transferred from the surrounding medium by the cells of the proximal convoluted tubules and concentrated in their lumina, Chambers and Cameron (1932), Chambers and Kempton (1933), Cameron and Chambers (1938). In a properly prepared explant, the proximal can easily be distinguished from the distal tubules (not shown in figures) even in the absence of an indicator. In cultures of 1- to 3-days incubation the cells of the proximal tubules are cuboidal and granular (Fig. 26a), while those of the distals and the collecting ducts are clear and hyaline. In older cultures when the proximal tubules have become markedly distended with secreted fluid (Fig. 26b), the cells are stretched thin and lose their cuboidal shape. Practically no change occurs in the appearance of the cells of the nondistended distal tubules.

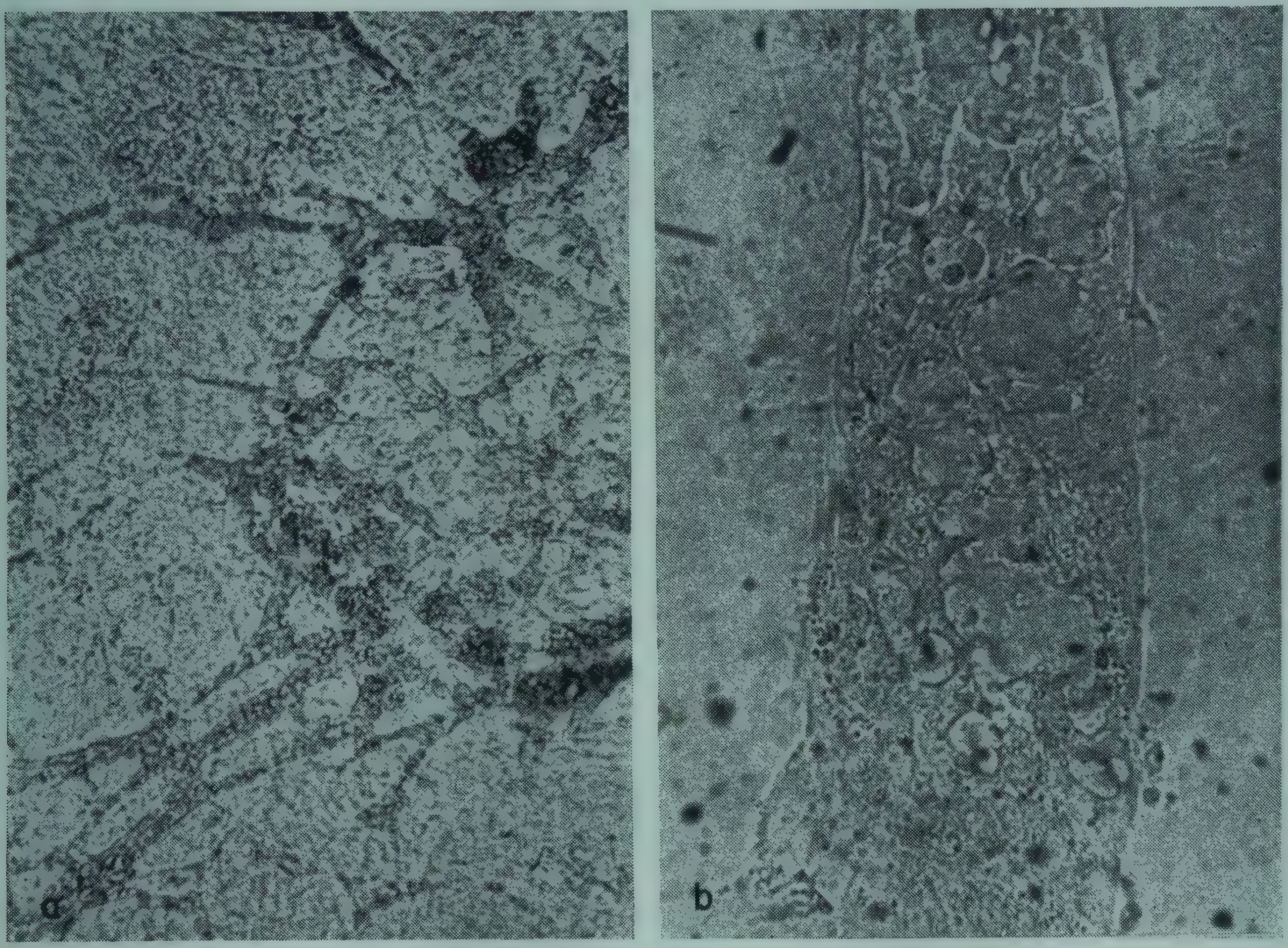


FIG. 25a. Living explant of chorio-allantoic membrane of 9-day chick embryo with blood vessels developing and extending. 4 days' growth. 100×.

FIG. 25b. Living culture of pancreatic duct extending from explant (not shown). The lumen is being developed by the formation of cysts. 7 days' growth. 250×.

A classic example of the activity of tissue explants is the continuation or initiation of contraction of cardiac muscle. Even after many weeks of culturing, contraction of individual muscle cells may be observed. With the gradual spreading of the tissue in the original explant the tissue becomes less dense so that the peculiar network of anastomosing fibers may be easily distinguished. The nuclei of the component cells are usually round and may be seen scattered throughout the fibers at irregular intervals. In cultures of several weeks, cross striations can be identified. This has been described by Goss (1933) in cardiac muscle of 16-day rat embryos, both in the muscle fibers in the explant and the outgrowth after several weeks of cultivation. Lewis (1926) and Hogue (1937), using 8-day chick embryos also observed cross striations and contraction for as long as 3 months.

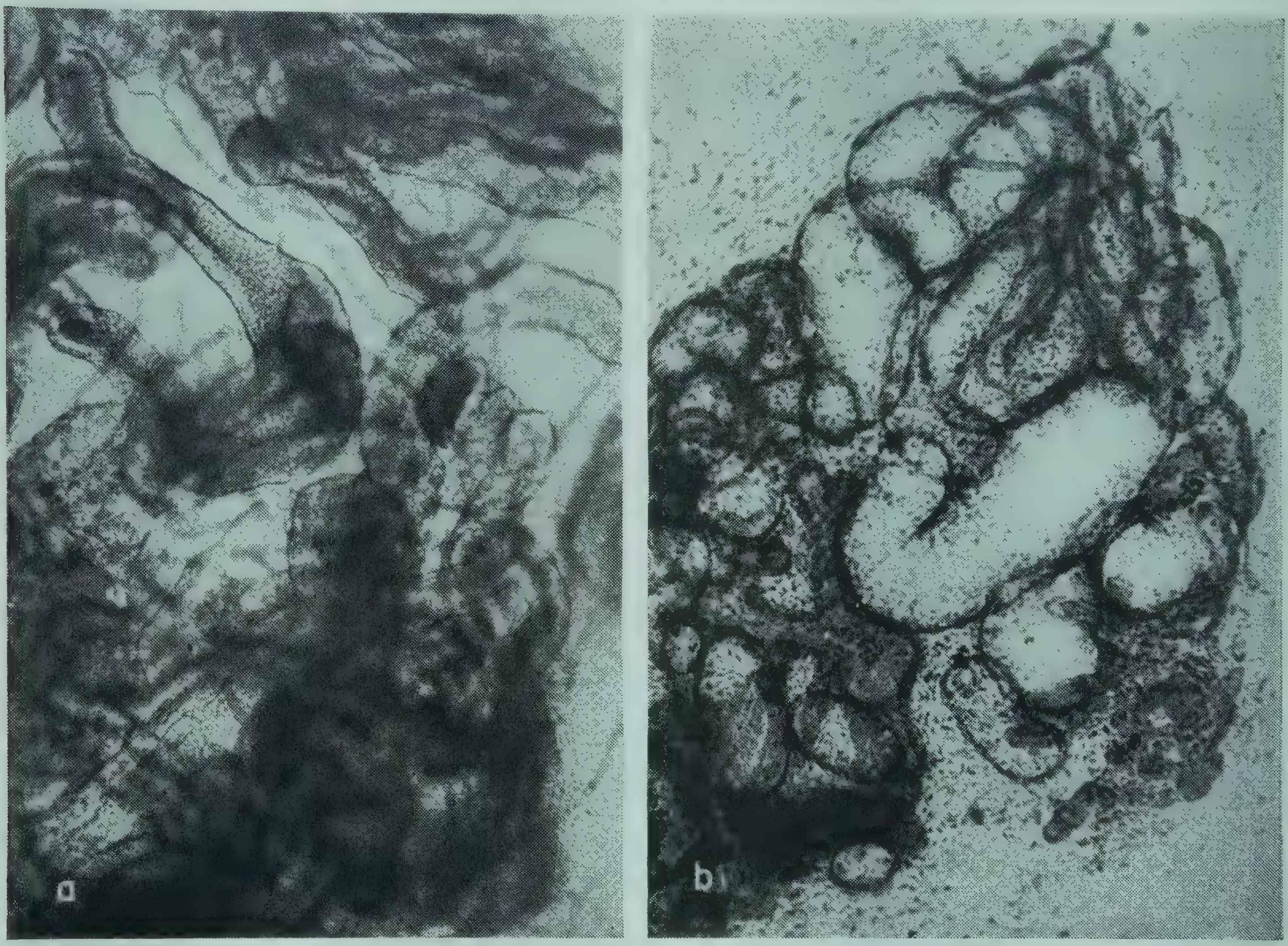


Fig. 26a. Living explant of mesonephros of 9-day chick embryo. No distension of lumina. 24 hours' growth. 100×.

Fig. 26b. Culture of mesonephros of 9-day chick embryo. Distension of lumina with secreted fluid. 72 hours' growth. 100×.

Contraction of embryonic skeletal muscle and rhythmic peristalsis of embryonic intestinal muscle may be observed, but identification of the individual muscle cells in the explant is difficult.

Strangeways and Fell (1926) and Fell (1939) have studied the development of explants of numerous still undifferentiated tissues such as limb buds, optic cup, otocyst, etc., and observed the differentiation of cartilage and bone, eye, ear, etc.

Young chick embryos (18- to 24-hours incubation) mounted according to Fell's method in watch crystals make simple and beautiful classroom preparations for study of the development of the explanted primitive streak.

The study of explants of some cold-blooded forms is also profitable. Special cases are melanophores and xanthophores in explanted fragments of amphibian, fish and squid skin, which remain active for long periods. We have maintained fragments of adult squid skin for 5 to 6 weeks and throughout that period have observed the contraction and expansion of the varicolored chromatophores through the action of the muscle cords attached to their margins. In skin from squid embryos, the development of pigment in the chromatophores may be studied.

CELLULAR OUTGROWTHS

The types of cells fall into several categories; wandering cells, fibrocytes and epithelial cells. Others which should be mentioned are muscle and nerve. In tissue culture all of these may be grown from both normal and malignant sources.

NORMAL CELLS

Wandering Cells

Generally speaking the term, wandering cells, is used to designate those cells that are not fixed in position, but move freely from place to place. Those that one sees most frequently in tissue cultures include granulocytes, lymphocytes, monocytes, macrophages and histiocytes. These migrate from the explant as individuals and usually remain as isolated cells. Wandering cells are particularly abundant in cultures of buffy coat (the layer of white blood cells lying on the erythrocytes of centrifuged blood), fragments of spleen (especially of embryos) and of lymph nodes.

In cultures of the *buffy coat,* (see Chapter IX), the wandering cells begin to migrate as early as one hour after incubation, after the culture has become thoroughly warm. The majority of these early appearing and actively ameboid cells are the granulocytes (Fig. 27), which, within a few hours, form a pronounced halo around the explant. The halo progressively increases in size and, after 24 hours, may cover an area many times larger than the explant itself. The granulocytes will then be found in large numbers at the periphery of the zone of migration and, closer to the explant, some large mononuclear leucocytes (monocytes) will be seen. As reported by Carrel and Ebeling (1922) the granulocytes disappear after about 10 days cultivation and a pure culture of monocytes remains.

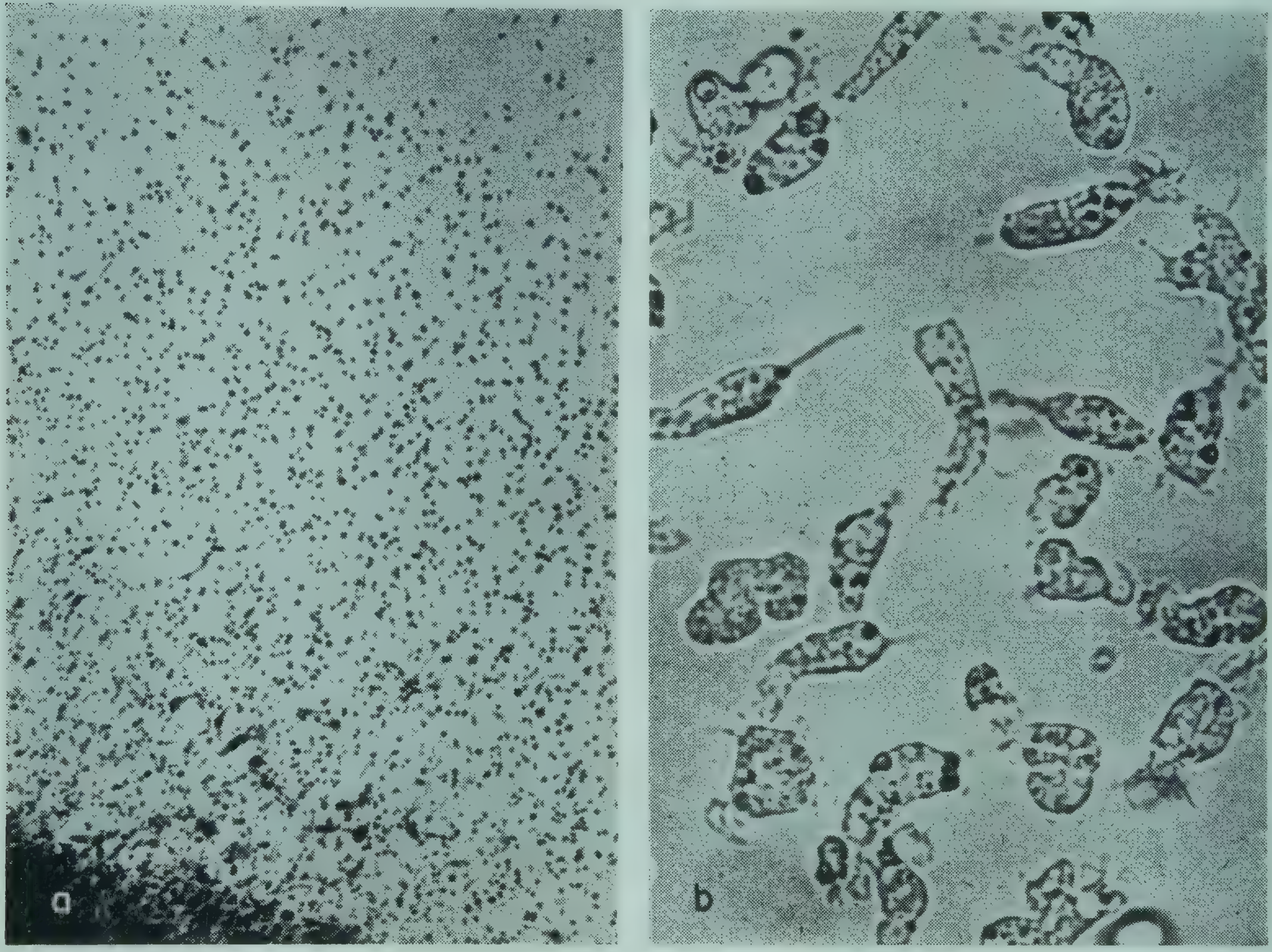
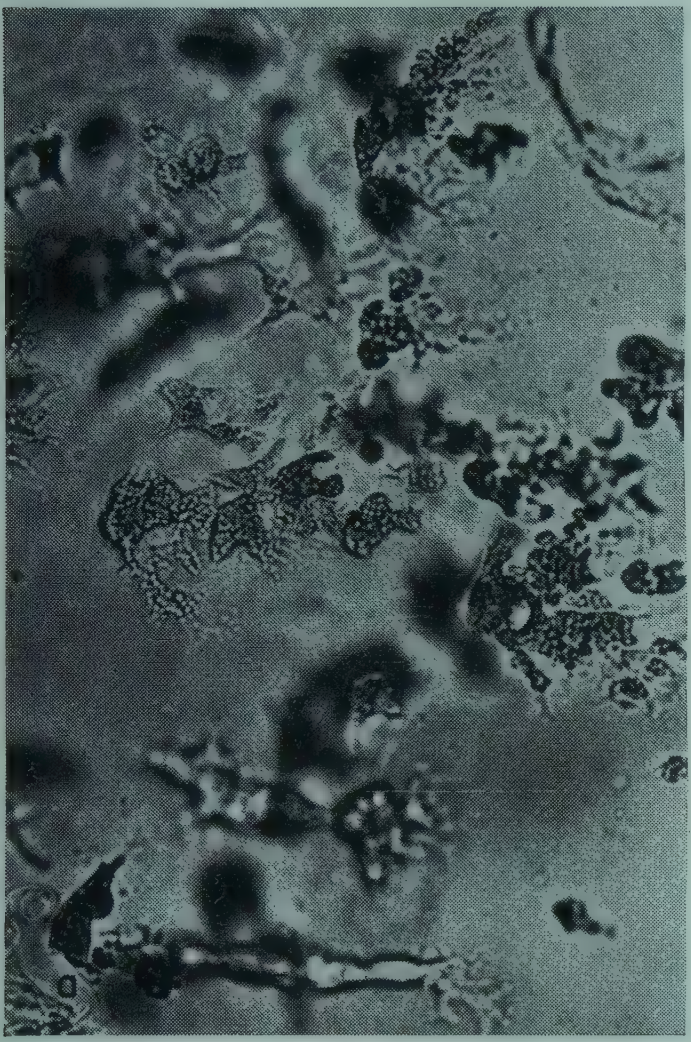


FIG. 27a. Granulocytes from centrifuged chicken blood. A few deeply stained larger cells, macrophages, close to explant. 24 hours' growth. Fixed and stained. 100×.
FIG. 27b. Living granulocytes from same culture (before fixation). 500×.

The growth of *spleen* resembles that of buffy coat during the first 24 hours. Granulocytes migrate actively in large numbers followed by monocytes. Small lymphocytes with clear nongranular cytoplasm and comparatively large round nuclei then appear. The lymphocytes frequently assume the "hand mirror" shape. They move more slowly than the granulocytes, usually with a crawling motion by means of one small pseudopod. Some macrophages or histiocytes (Fig. 29b), will be seen at intervals throughout the area of migration. These are much larger than the granulocytes and have a large eccentric nucleus or may be multinucleated. The cytoplasm contains granules, vacuoles and, frequently, phagocytosed inclusions. Macrophages are markedly ameboid, moving by means of ever-changing elongated pseudopodia. If heavily laden with ingested material, the cell becomes sluggish, the pseudopodia are withdrawn and the cell becomes rounded. Frequently mitotic divisions may be seen. A few fibroblasts will begin to emerge from the explant after 24 to 36 hours incubation. Figure 28a shows Kupffer cells, the macrophages of the liver, and Figure 28b reticular cells (macrophages) from a human lymph node.



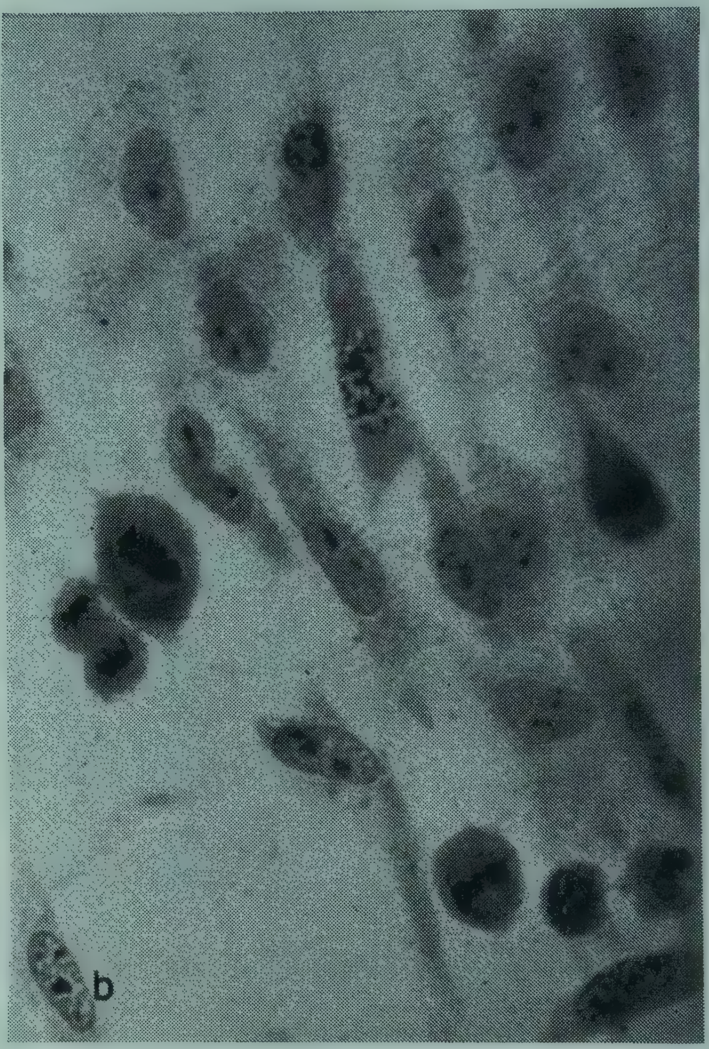
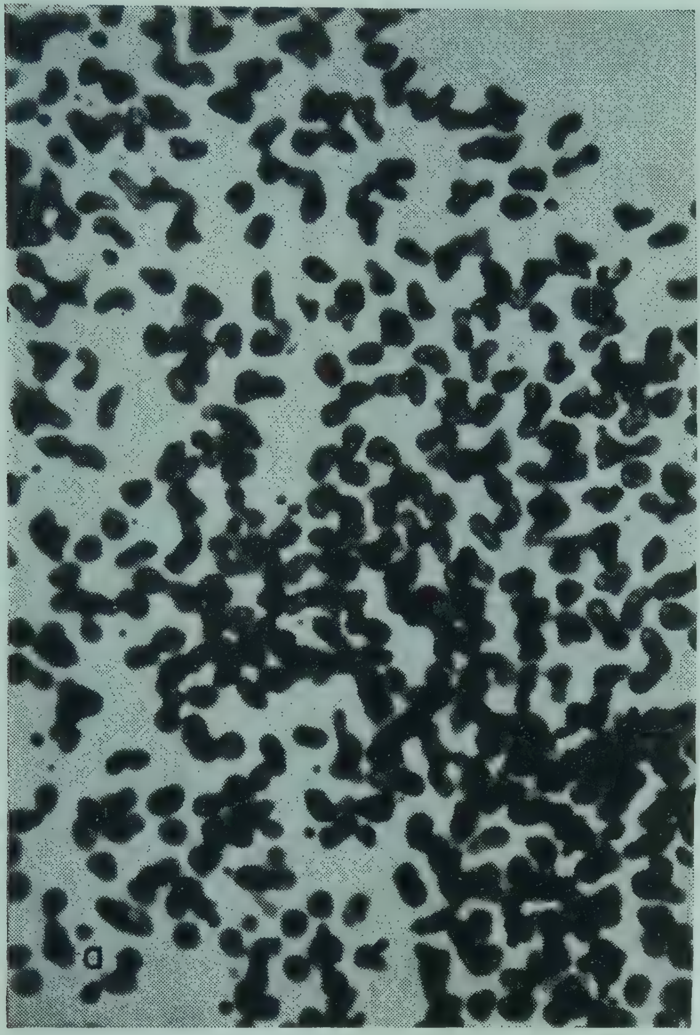


Fig. 28a. Kupffer cells from living culture of adult rat liver. 48 hours' growth. 300×.

Fig. 28b. Reticular cells (macrophages) from culture of human lymph node showing six cells in various stages of mitoses. Fixed and stained. 400×.

From fragments of *lymph nodes* the migrating cells will be predominantly lymphocytes (Fig. 29a). A few granulocytes and some histiocytes or reticular cells will also migrate within 24 hours followed at about 36 hours by fibroblasts and possibly some endothelium from blood vessels. In older cultures, especially those grown in fluid medium where the cells use the surface of the coverslip for support, some lymphoid cells may become thin, flattened and inactive and frequently multinucleated. They have been designated epithelioid cells.

Some types of wandering cells are present in explants of almost all fresh tissue and these are the first to migrate into the medium. Granulocytes, when present, will appear first, then lymphocytes and macrophages. These are followed by fibroblasts, epithelium, muscle, etc.



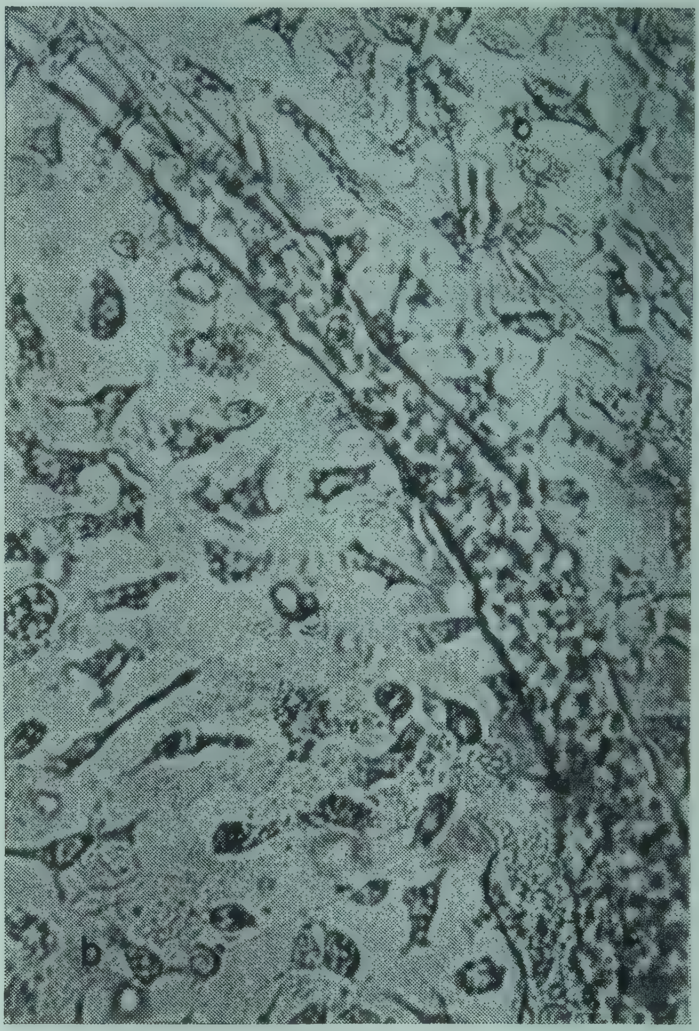


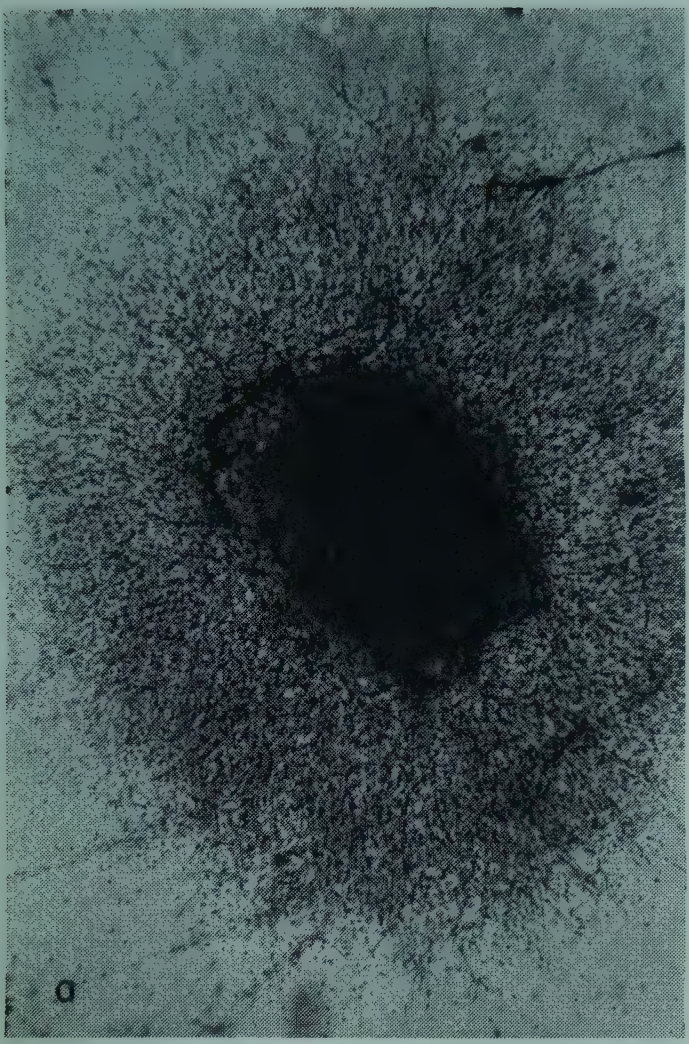
Fig. 29a. Lymphocytes from culture of adult mouse lymph node. Fixed and stained. 250×.

Fig. 29b. Macrophages from living culture of mouse carcinoma. A tongue of epithelium is also shown. 48 hours' growth. 200×.

Fibrocytes

The term *"fibrocyte"* has been introduced to replace the name fibroblast. It is a more general term to designate cells both of embryonic and of adult connective tissue.

Fibrocytes are present in nearly all mixed cultures, where they grow and proliferate readily unless special precautions (see Chapter XI) are taken to inhibit their proliferation. The typical fibrocyte, whatever its source, is spindle shaped, with clear cytoplasm containing a few granules and an oval nucleus. When growing actively, fibrocytes form a reticulum with feathery periphery. Usually they are not in close contact with one another and do not adhere to one another along their lateral surfaces



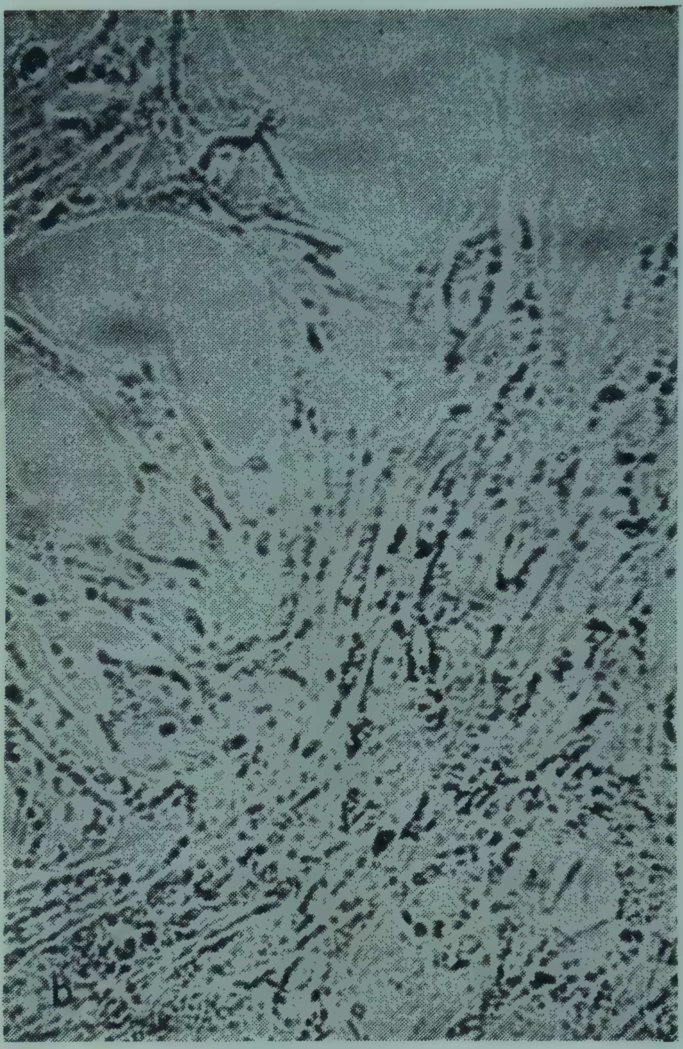
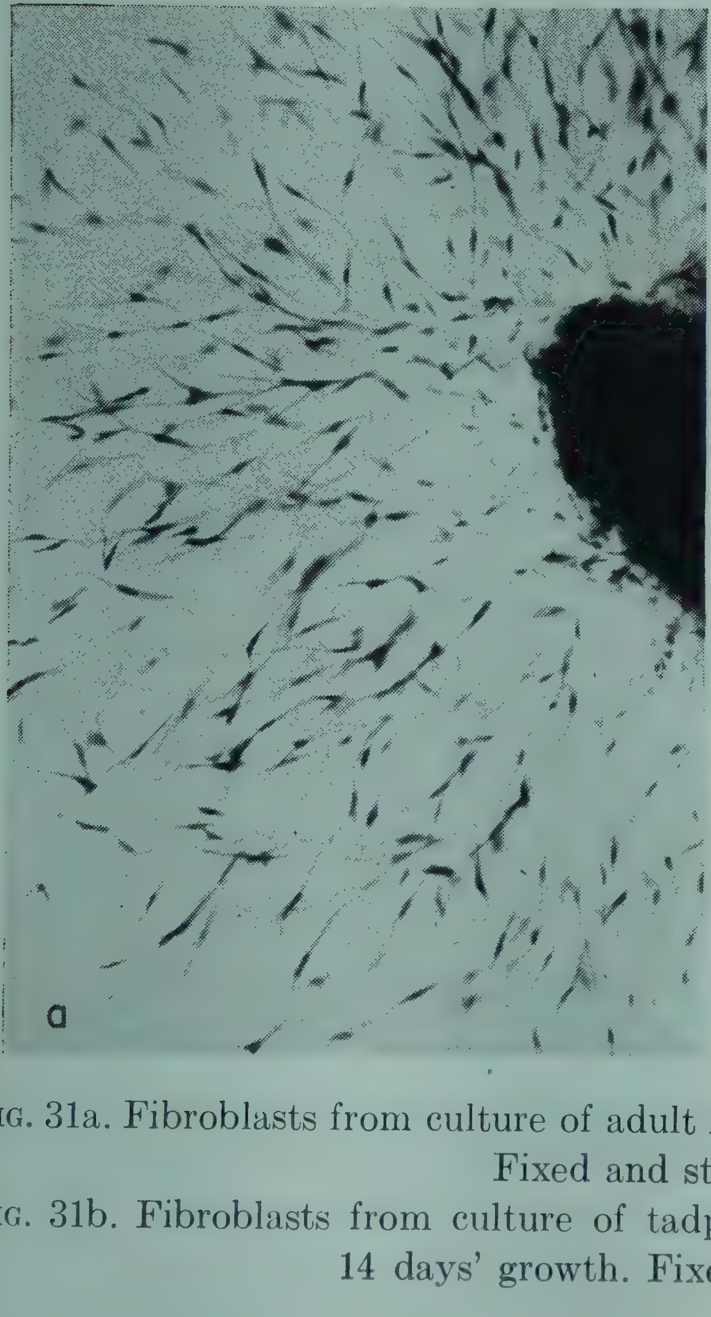


FIG. 30a. Fibroblasts from culture of 9-day chick embryo heart. 48 hours' growth. Fixed and stained. 32×.

FIG. 30b. Fibroblasts from similar culture. Living. 200×.



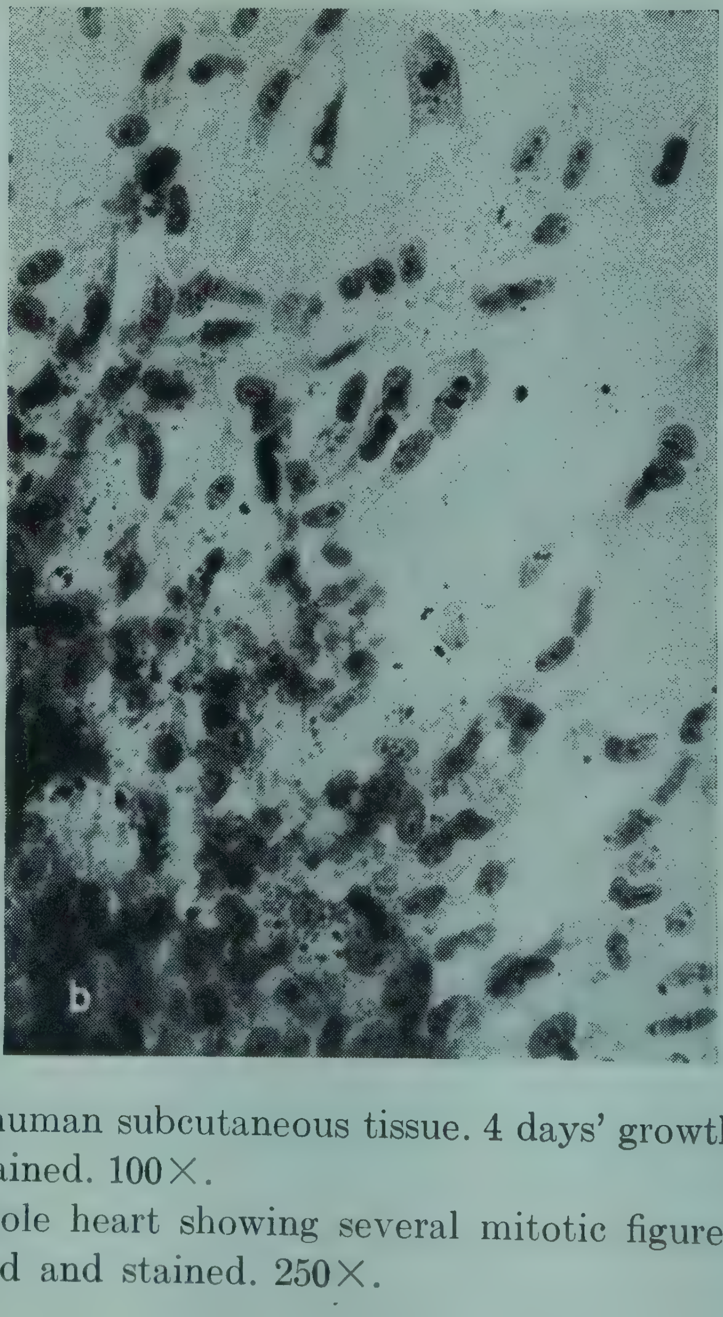


FIG. 31a. Fibroblasts from culture of adult human subcutaneous tissue. 4 days' growth. Fixed and stained. 100×.

FIG. 31b. Fibroblasts from culture of tadpole heart showing several mitotic figures. 14 days' growth. Fixed and stained. 250×.

as in the case of epithelial sheets. Occasionally, fibrocytes at the margin of the growing network become separated and then resemble histiocytes. Figures 30 and 31 show fibroblasts of chick, human and amphibian origin.

Noncellular elements of connective tissue may sometimes be observed in cultures, such as *elastic, argyrophile* or *collagenous* fibers. Fat cells of adipose tissue may also be seen. *Cartilage* cells grow out into the medium relatively slowly. At first they appear as small round cells which might be confused with lymphocytes but later on tend to form psuedo sheets, the cells of which become somewhat spindle shaped. They may be distinguished from fibrocytes, however, by their large round nuclei.

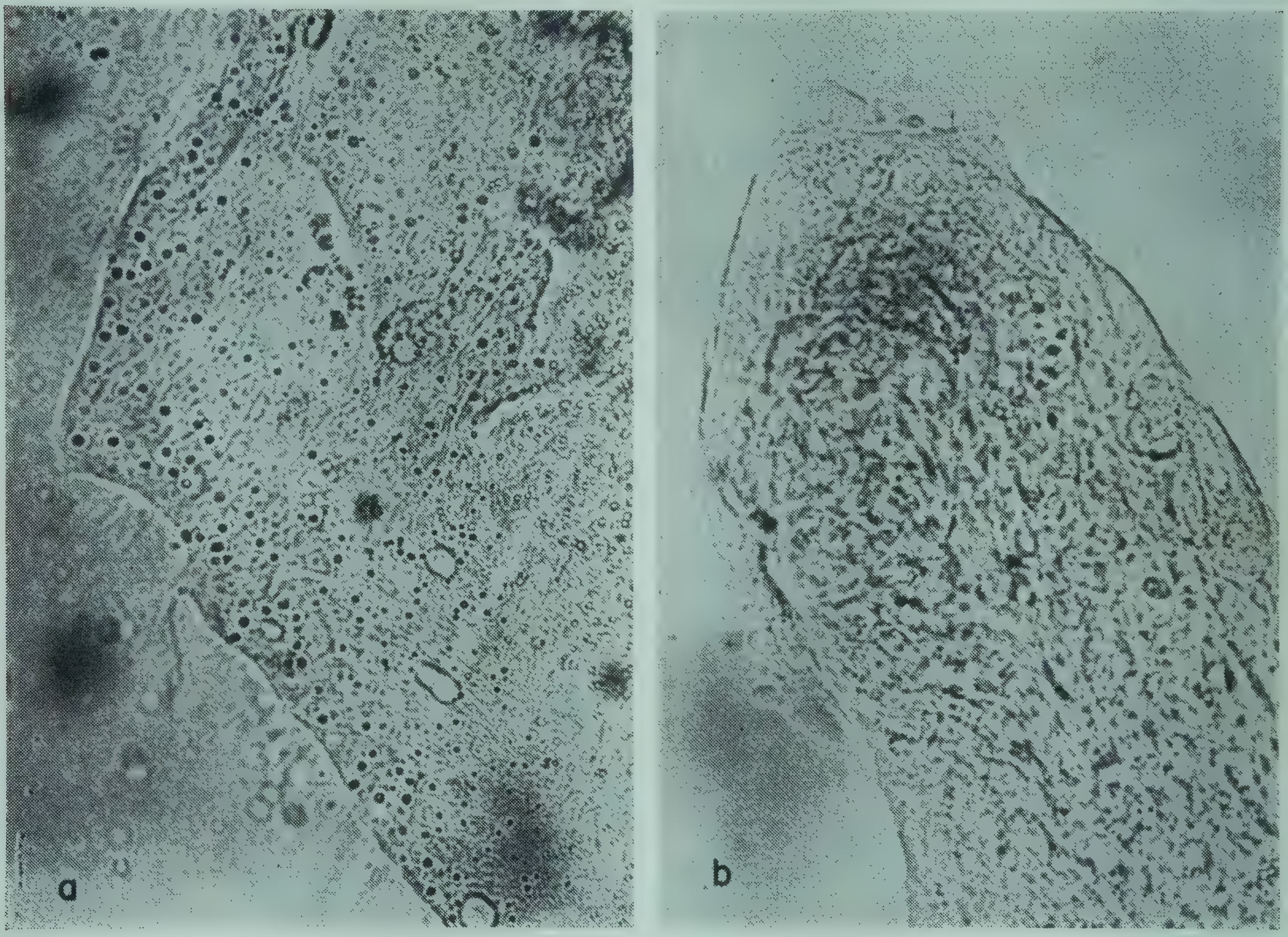
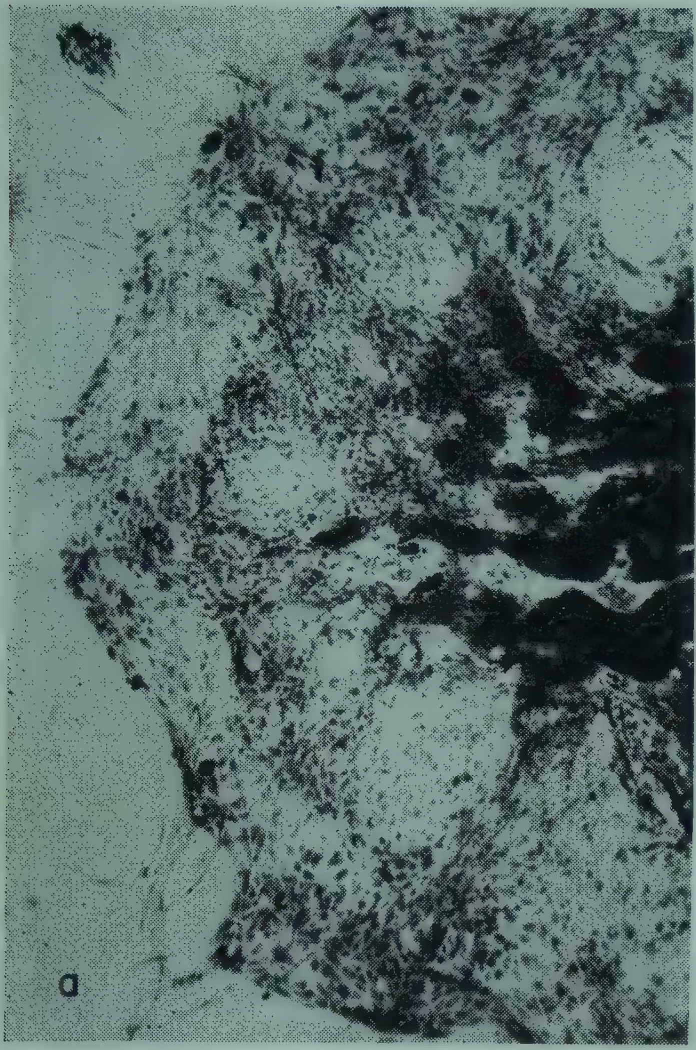


FIG. 32a. Epithelial sheet from living culture of intestine from 12-day chick embryo. Note typical smooth outline of periphery, fat droplets (black) and thickening of epithelium in glandular areas. 48 hours' growth. 250×.

FIG. 32b. Epithelial sheet from living culture of 15-day chick embryo tongue. 4 days' growth. 250×.

Epithelium

Epithelium is a comparatively slow growing tissue. When present in a mixed culture it tends to make its appearance in the outgrowth later than the other cells. Two characteristic types of epithelial growth may be obtained, compact sheets, or groups of cells organized into tubules or cysts. The cells lie in close apposition to one another and, when growing as a sheet, its boundary is smoother and more regular in outline than that of the fibroblastic network (Figs. 32, 33). In areas of organization the arrangement of the cells is similar to that of the tissue from which it was obtained, such as tubular formation in cultures of kidney, acini and follicles in those of mammary and endocrine glands respectively.



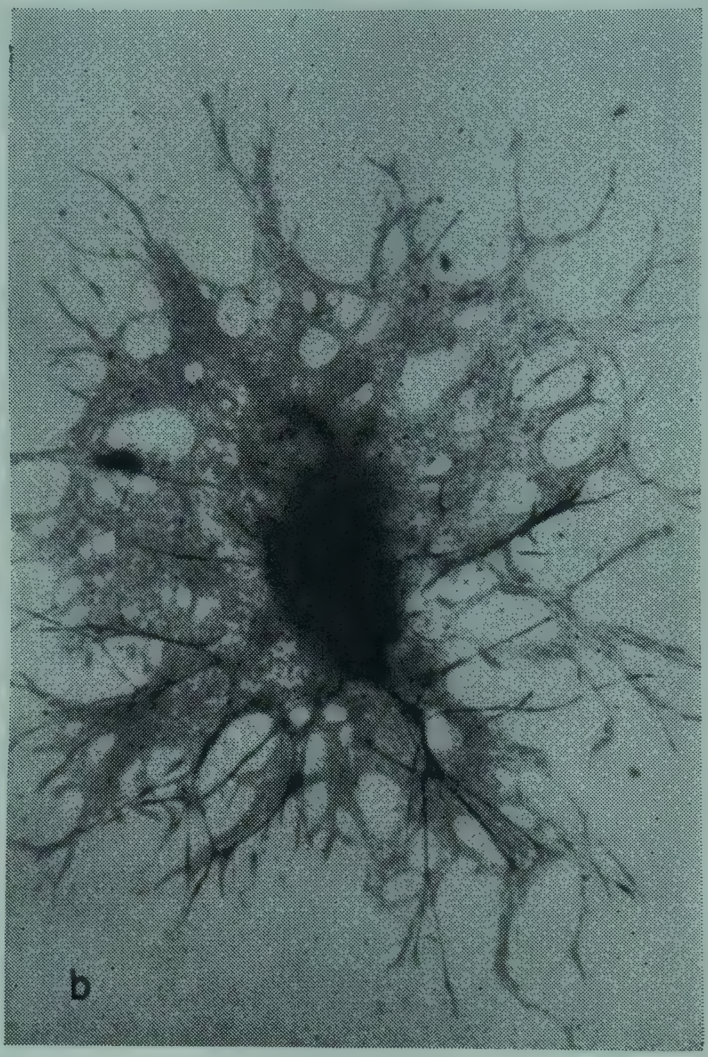


FIG. 33a. Epithelial sheets from fixed and stained culture of 9-day chick embryo mesonephros. A few fibrocytes at periphery. Tubules deeply stained. 6 days' growth. 100×.

FIG. 33b. Explant and epithelial outgrowth of 8-day chick embryo iris, showing development of pigment. Living 72 hours' culture. 50×.

Squamous, cuboidal and columnar epithelium may be grown and identified in tissue culture. Squamous epithelium grows in thin sheets, usually one cell thick. Normally the cells are relatively large, polygonal in shape, regular in outline and contain ovoid nuclei. This type of epithelium may grow from the smaller excretory ducts of some of the glands or from the endothelial lining of blood vessels, etc. Cuboidal and columnar epithelium from many of the glands may show some typical organization and orientation on a basement membrane. These may give evidence of secretory activity, Cameron (1948). In other regions, the epithelium grows in flattened sheets that are usually somewhat denser and the cells more granular than those of squamous epithelium. Most normal epithelial cells each have one round nucleus. An exception to this is seen in the glandular cells of the liver, which frequently contain two or more nuclei. Transitional stratified epithelium, such as is found in the urinary bladder, will grow and develop characteristic structures, but most of them eventually tend toward the pavement sheet-like type of growth. Sheets of epithelium from the retina or iris (Fig. 33b), and from the skin of amphibians (Figs. 34, 35b), may contain pigment granules. In cultures of the superficial epithelium of various cold-blooded forms cilia may be observed that often continue to beat for periods up to several weeks.

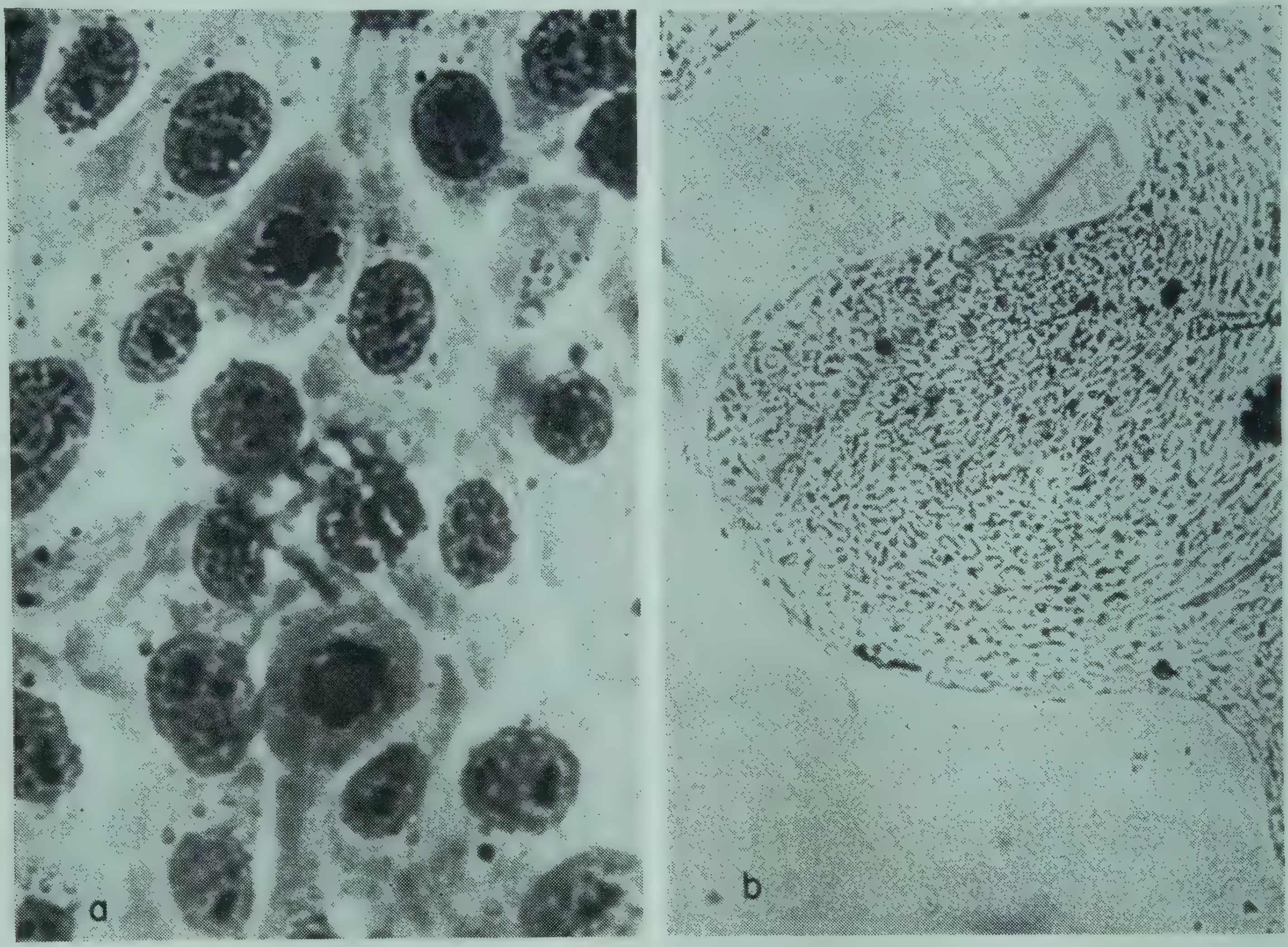
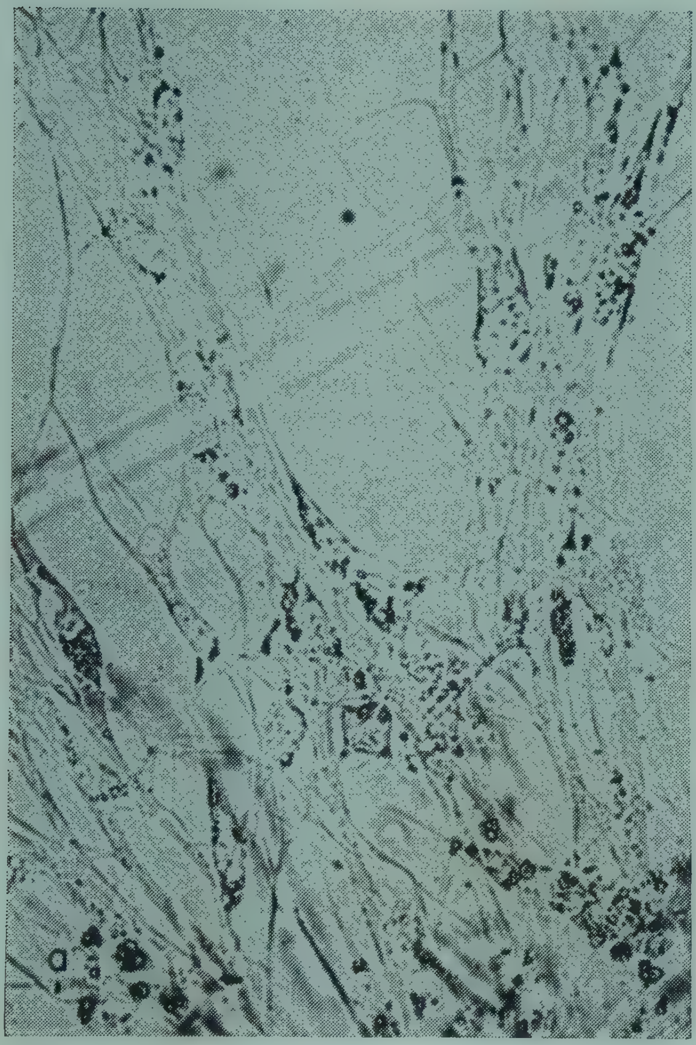


FIG. 34a. Epithelium from culture of tadpole epidermis. Note mitotic figures. 5 weeks' growth. Fixed and stained. 600×.

FIG. 34b. Epithelium from living culture of tadpole epidermis. Note presence of pigment in cells and their swirling arrangement. 6 weeks' growth in roller tube. 200×.

Muscle

Embryonic *muscle* cells readily grow and differentiate in tissue culture. Cardiac muscle cells usually first appear at the edge of the explant as isolated, relatively clear spindle shaped cells, which may or may not show contraction. Then groups or buds of cells may be seen which finally form anastomosing bands surrounding the explant and extending into the medium. Fibrocytes will also be present but the muscle fibers are easily distinguished and tend to lie closer to the coverslip than the fibroblastic network. In older cultures, those maintained one to several months, there may be observed cross striations and myofibrillae in the outgrowing fibers as well as in those of the explant.



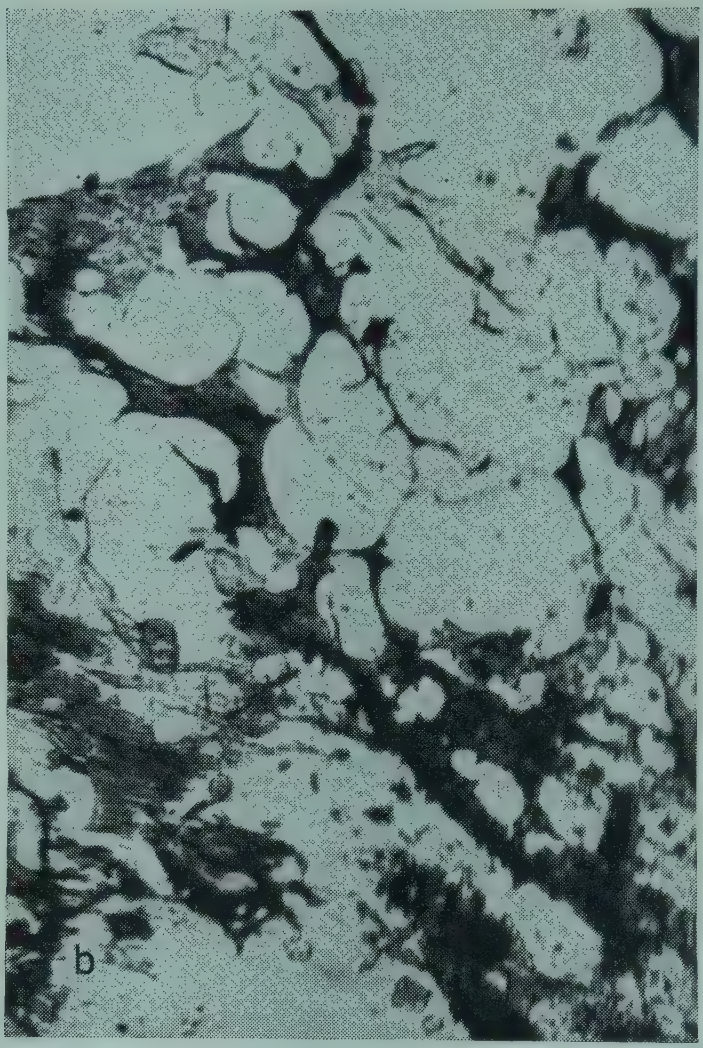


FIG. 35a. Neuroblasts with neurofibrils and sheath cells from living culture of spinal cord of tadpole. 14 days' growth. 200×.

FIG. 35b. Chromatophores from living culture of tadpole skin. 3 weeks' growth in roller tube. 200×.

Embryonic skeletal muscle also grows luxuriantly (Fig. 36b). At first the cells appear as individuals but soon form extensive radiating bands of muscle fibers. These fibers are irritable, respond to light, and to mechanical and electrical stimuli.

Smooth muscle cells are not easily identified in living cultures. They are spindle shaped with a fairly homogenous cytoplasm and a long oval nucleus.

Nervous elements have been cultivated ever since Harrison's studies on the developing nerve fibers from the neural tube of the frog embryo.

Brain (Fig. 36a) and spinal cord (Fig. 35a) of chick, mammalian and amphibian embryos grow quickly in tissue culture and provide interesting demonstrations of neuroglia and of nerve cells. The nucleus of the cell body of neurons can be recognized as comparatively large, round and centrally located. The cells have many thread-like processes. Within the cell neurofibrils and mitochondria have been observed in the living cultures. Murray and Stout (1942a,b, 1947a,b) describe the characteristics of human Schwann cells and sympathetic ganglion cells, Weiss (1934) the factors determining the course of outgrowing nerve fibers, Weiss and Taylor (1946) the effect of methylene blue on isolated nerve fragments, and Lewis (1945) axon growth and regeneration.

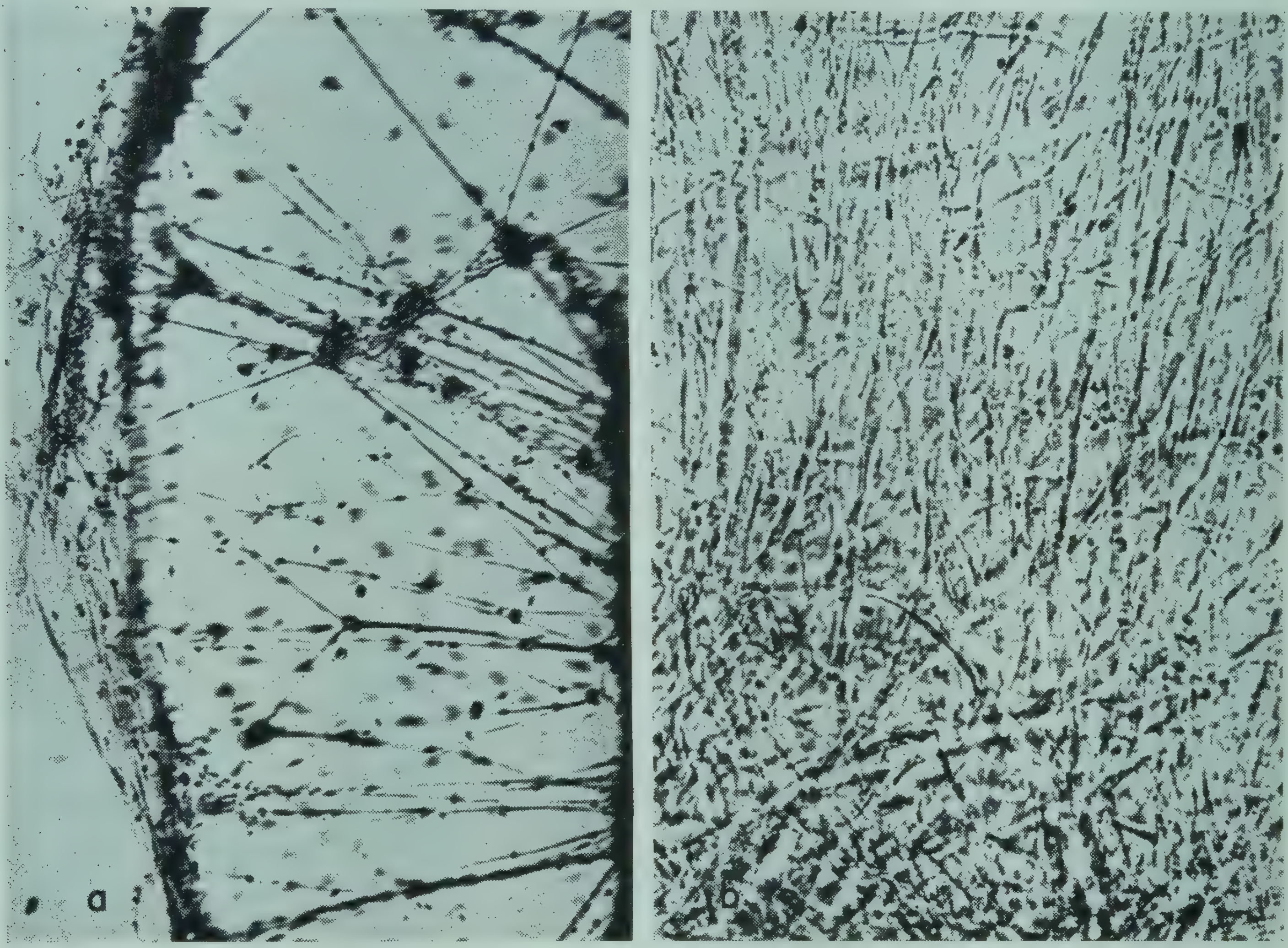


Fig. 36a. Living culture of brain from 10-day chick embryo. Typical growth of neurofibrillae and neuroglia across liquefied area. Explant at right. 72 hours' growth. 100×. Fig. 36b. Muscle fibers interspersed with fibroblasts in living culture of 10-day chick embryo pectoral muscle. Explant at base. Periphery not shown. 150×.

NEOPLASTIC TISSUES

A few characteristics of a more or less general nature may be mentioned as well as the broad classifications of some of the most frequently found tumors, such as lymphomas, sarcomas and carcinomas.

The malignant cell migrates quickly in tissue culture, multiplies rapidly, sometimes by multipolar mitosis. Irregularity and abnormal arrangement of the chromosomes is characteristic of many malignant cells with resulting multinucleated cells or aberrant mitoses and daughter cells of unequal size.

Most actively growing tumors have the ability to liquefy a coagulated plasma medium. This proteolytic activity is markedly in excess of that possessed by normal epithelium and has been used as a criterion of the degree of malignancy.

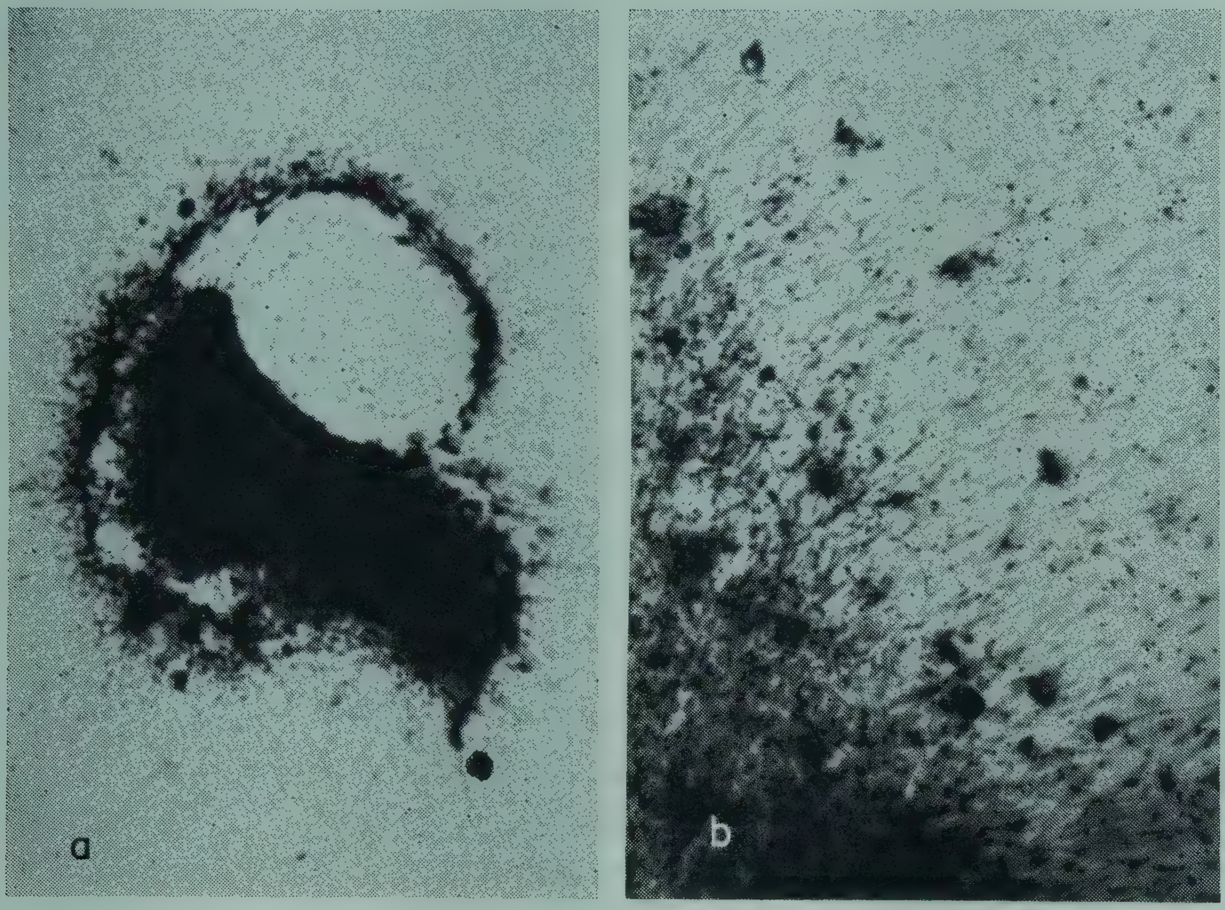


Fig. 37a. Whole culture of tissue from Hodgkin's disease lymph node. 48 hours' growth. Note areas of liquefaction. Fixed and stained. 55×.
Fig. 37b. Higher power of similar culture showing growth of fibrocytes, lymphocytes and the deeply stained giant cells. 160×.

Lymphomas

Most lymphomas such as lymphosarcomas, reticulo-cell sarcomas, lymphatic leukemias and Hodgkin's disease nodes grow luxuriantly in culture. The lymphoid cells from fragments of lymphosarcoma begin to migrate after a few hours incubation and tend to be irregular in size and shape. Fibrocytes (stroma cells), begin to appear in the outgrowth after about 24 hours incubation. At this period liquefaction will probably commence and be first observed immediately surrounding the explant. If the liquefaction is excessive, the lymphocytes in the area become rounded and tend to form clumps of cells. Later, the clumps may adhere to the coverslip and the cells then become flattened and epithelioid. We have grown many cultures of Hodgkin's disease lymph node (Figs. 37, 38) in this laboratory as described by Grand (1944, 1949). The lymphocytes appear first, followed quickly in active cases of the disease by liquefaction of the medium around the explant. The lymphocytes exhibit marked degeneration. No mitoses are observed such as are seen in cultures of lymphosarcoma. Sternberg-Reed cells are sluggish and usually do not appear in the culture medium until after about 48 to 72 hours. Their number and size increases with the age of the culture.

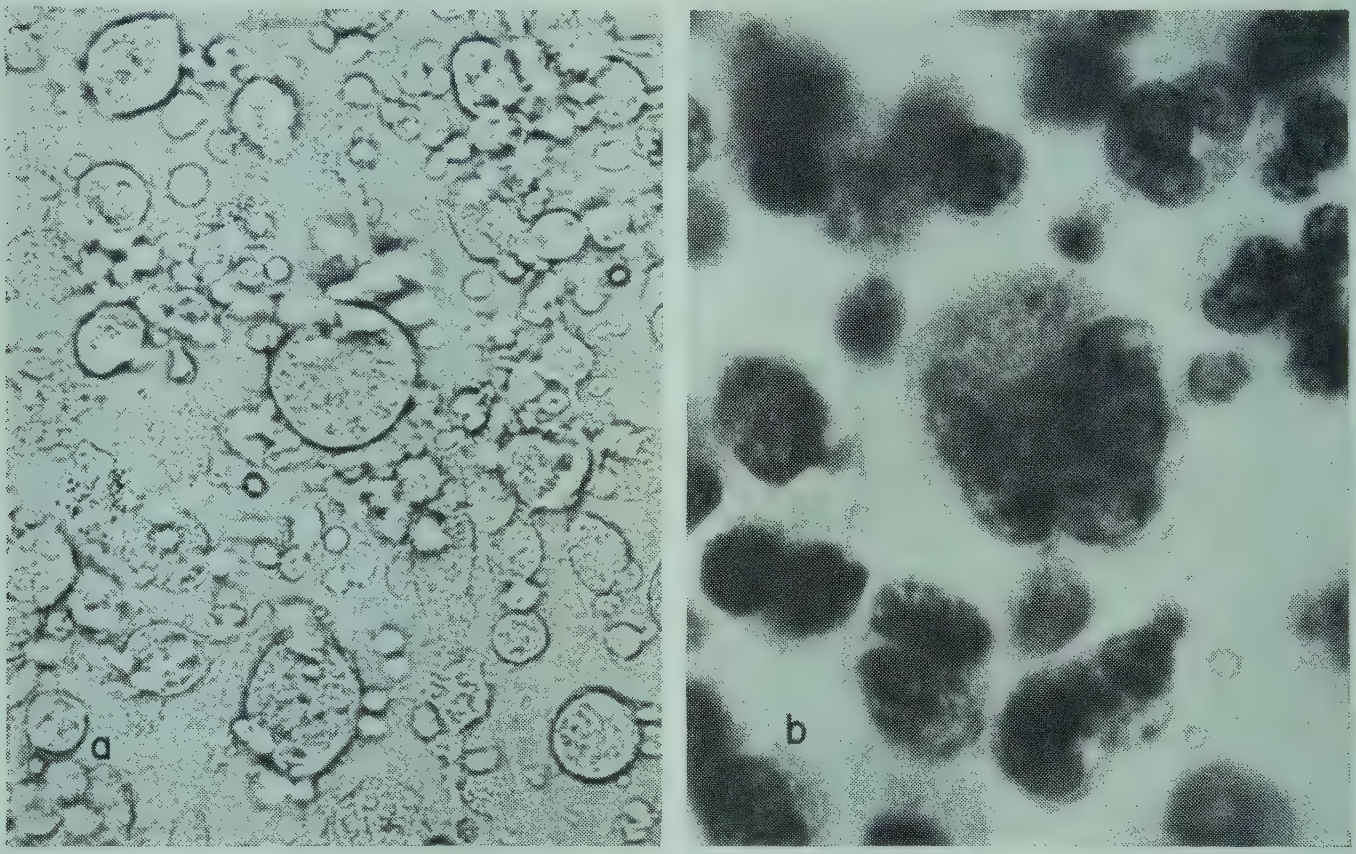
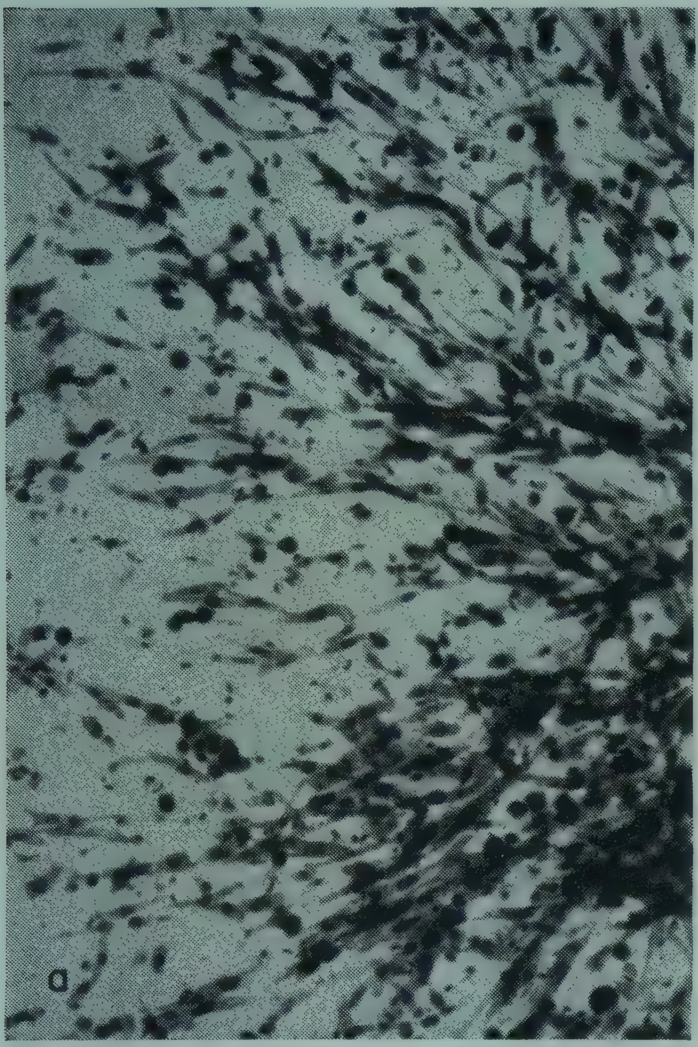


FIG. 38a. Giant cells and Sternberg-Reed cells from living culture of Hodgkin's disease lymph node. 72 hours' growth. 500×.
FIG. 38b. Similar cells surrounded by multinucleated reticular cells fixed and stained. 1200×.

Sarcomas

There are many types of sarcomas, one of the main groups of which is fibroblastic (Fig. 39a). The sarcoma cells usually begin to migrate from the explant much earlier than normal fibrocytes, after about 6 to 8 hours incubation. The cell is spindle shaped, wider in diameter, larger and more granular than the normal fibrocyte and with a relatively large nucleus and irregular nucleoli. Sarcoma cells possess marked proteolytic powers and in the liquefied areas become rounded, and unless fresh plasma is added may degenerate. Lewis (1935b) has described the cytological differences of the normal and malignant fibroblast.

Numerous strains of rat and mouse sarcomas have been maintained for several years in tissue culture by Lewis (1935b) and by Gey and Gey (1936) without observable changes or loss of malignancy, as attested by the formation of tumors similar to the originals when reinoculated into animals.



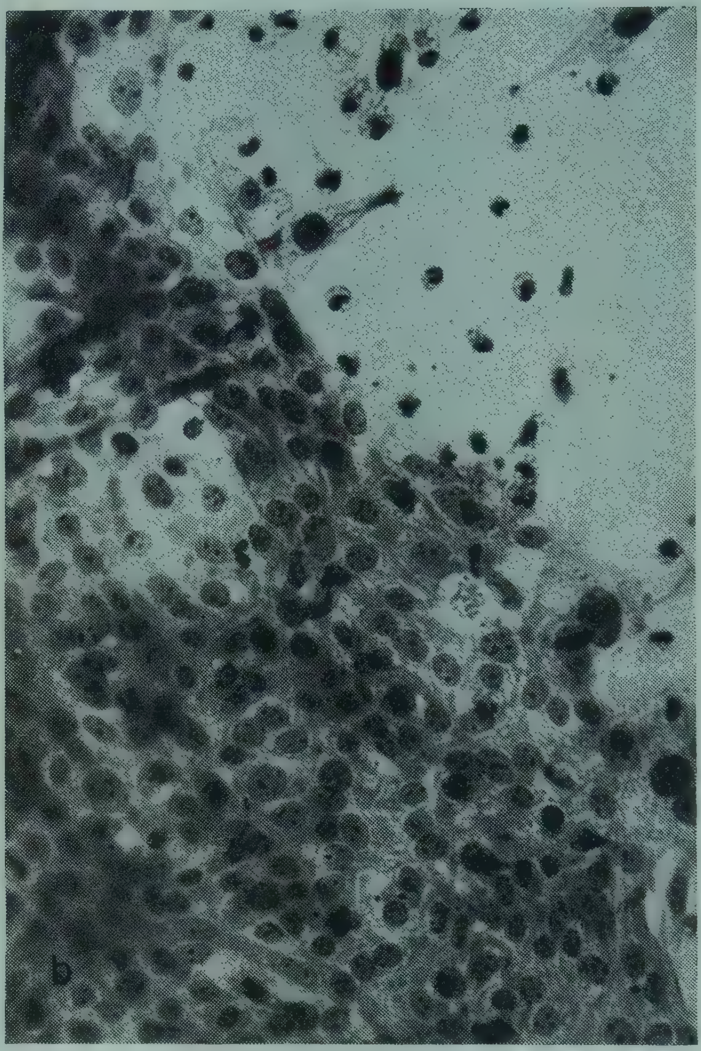


Fig. 39a. Culture of mouse sarcoma 180 showing spindle shaped sarcoma cells and macrophages. 72 hours' growth. Fixed and stained. 250×.

Fig. 39b. Culture of spontaneous mammary carcinoma from mouse, showing epithelial sheet and a few isolated macrophages. 48 hours' growth. Fixed and stained. 300×.

In this laboratory we have made an extensive study of human, mouse and fish melanomas (Grand, Chambers and Cameron, 1935; Grand, Gordon and Cameron, 1941; Grand and Cameron, 1948). The first cells to migrate, after about 12 hours, are macrophages laden with phagocytosed melanin granules (Fig. 41a). Some appear to drag the granules in the posterior portion of the cell while the cytoplasm of the advancing pseudopodia is relatively clear. The spindle-shaped fibrocytes appear next, after about 24 hours. They usually contain some melanin but much less than

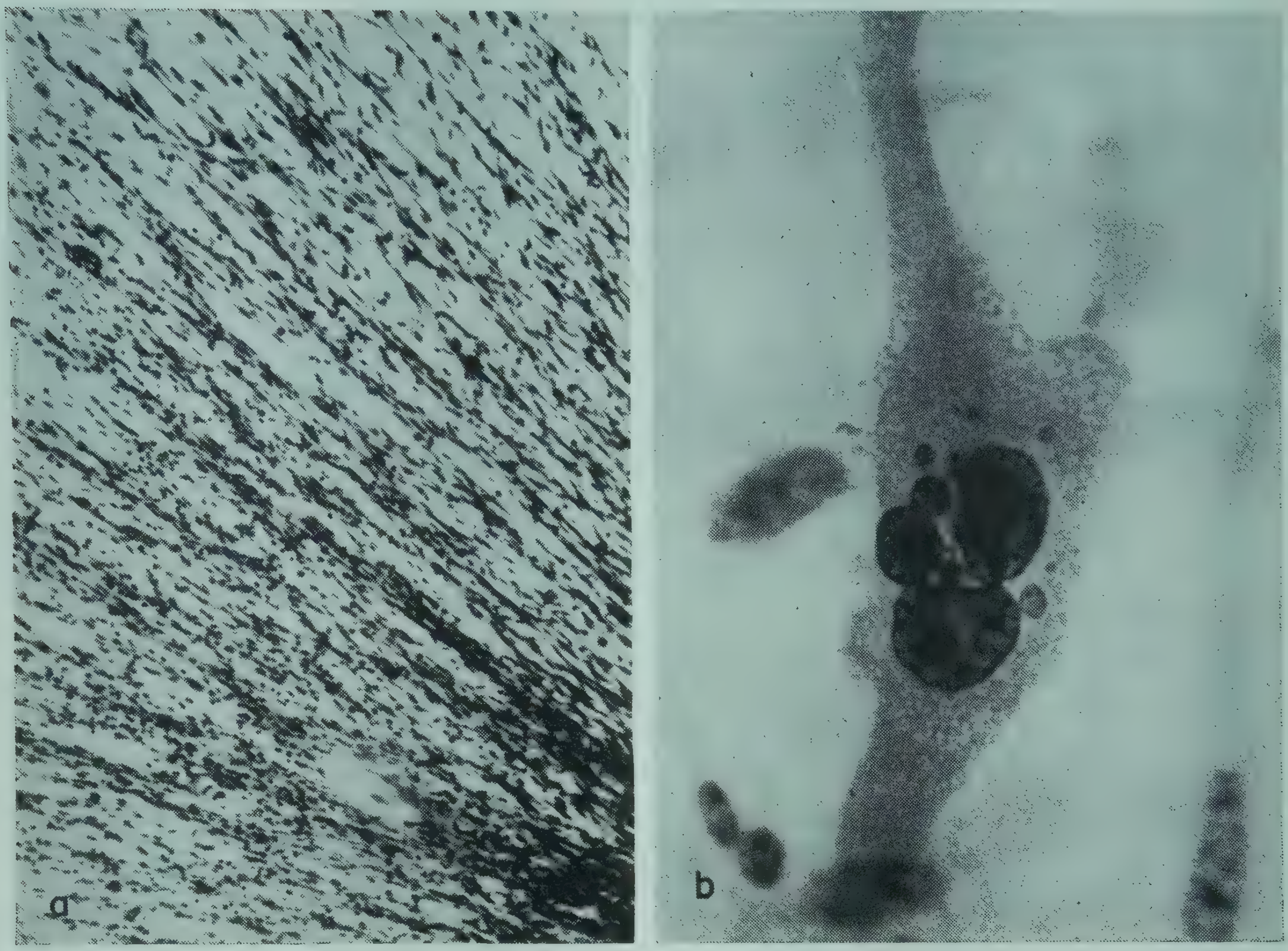


FIG. 40a. Myoblasts forming fibers from culture of rhabdomyoma of the mouse. Fixed and stained. 100×.

FIG. 40b. Giant cell from culture of rhabdomyoma. Fixed and stained. 1000×.

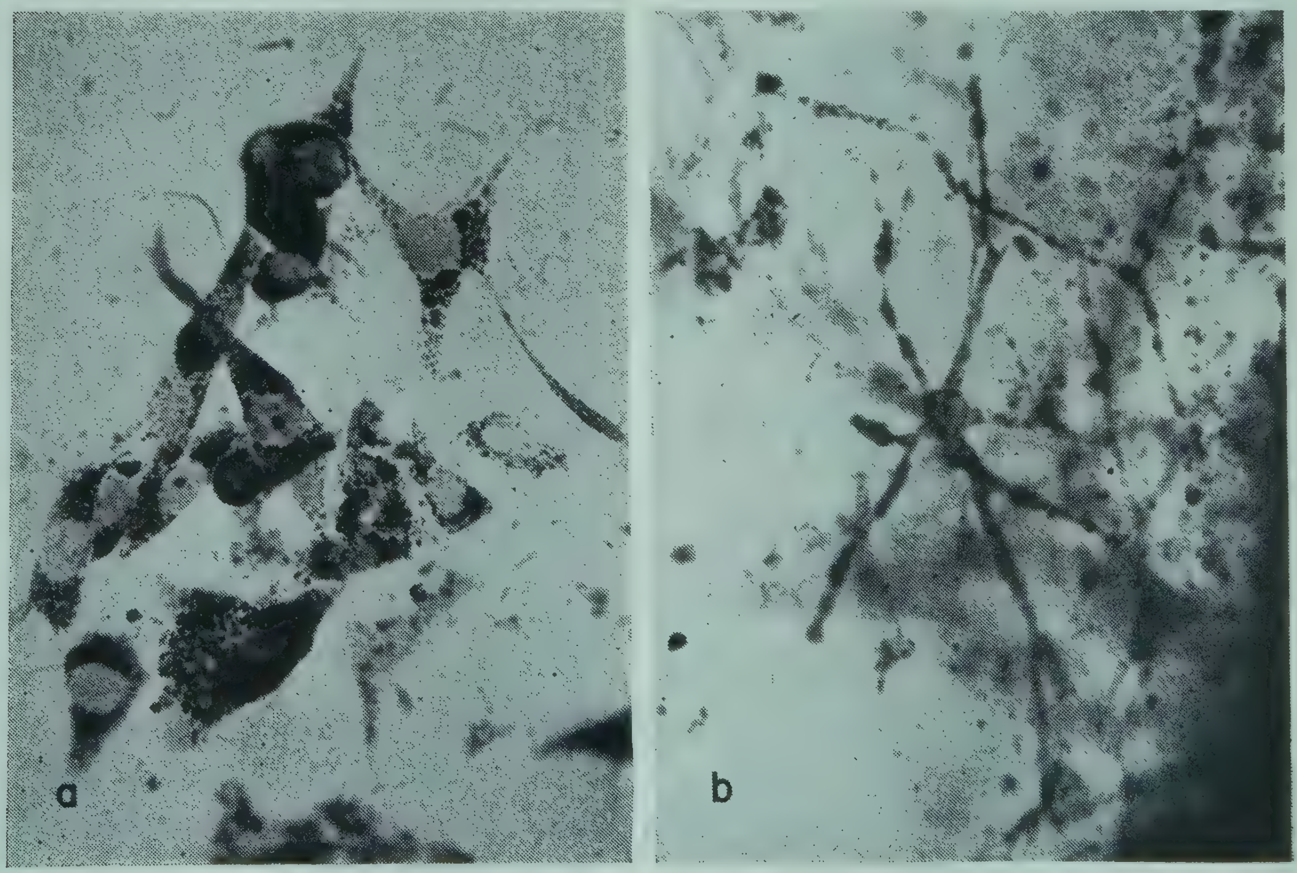


FIG. 41a. Macrophages from living culture of Harding and Passey mouse melanoma. 335×.

FIG. 41b. Melanoblast from a similar culture. 335×.

the macrophages, either in clumps or dispersed around the oval nucleus. The delicate, filamentous dendrites of the melanoblasts (Figs. 41b, 42b), may first be seen emerging from the margin of the explant after about 24 to 36 hours. The comparatively small cell body appears considerably later, usually after 72 hours incubation. These are the specific cells of the melanoma and were observed to eliminate melanin granules by clasmatosis. Melanophores of fish melanomas are shown in Figure 42a.

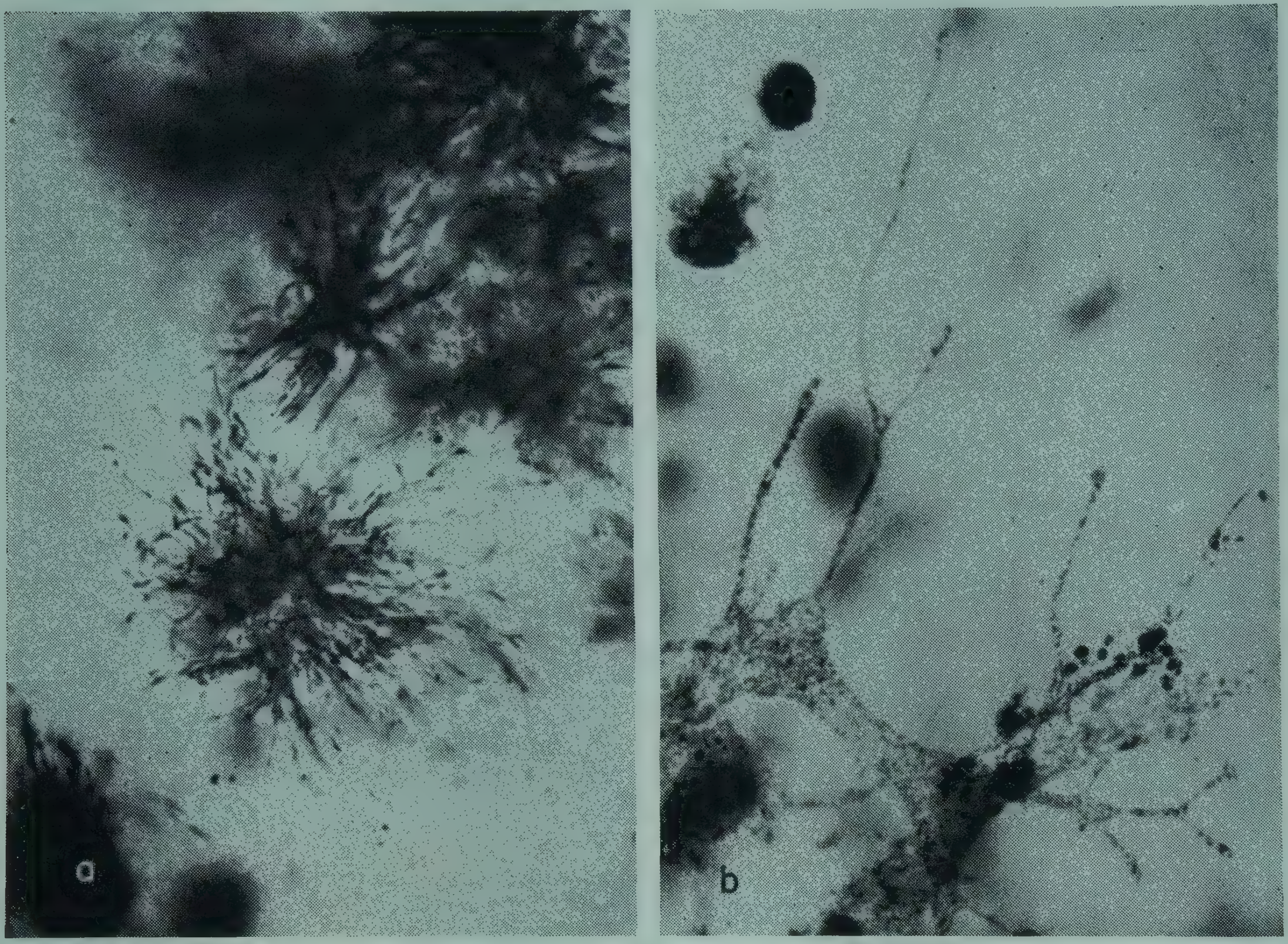
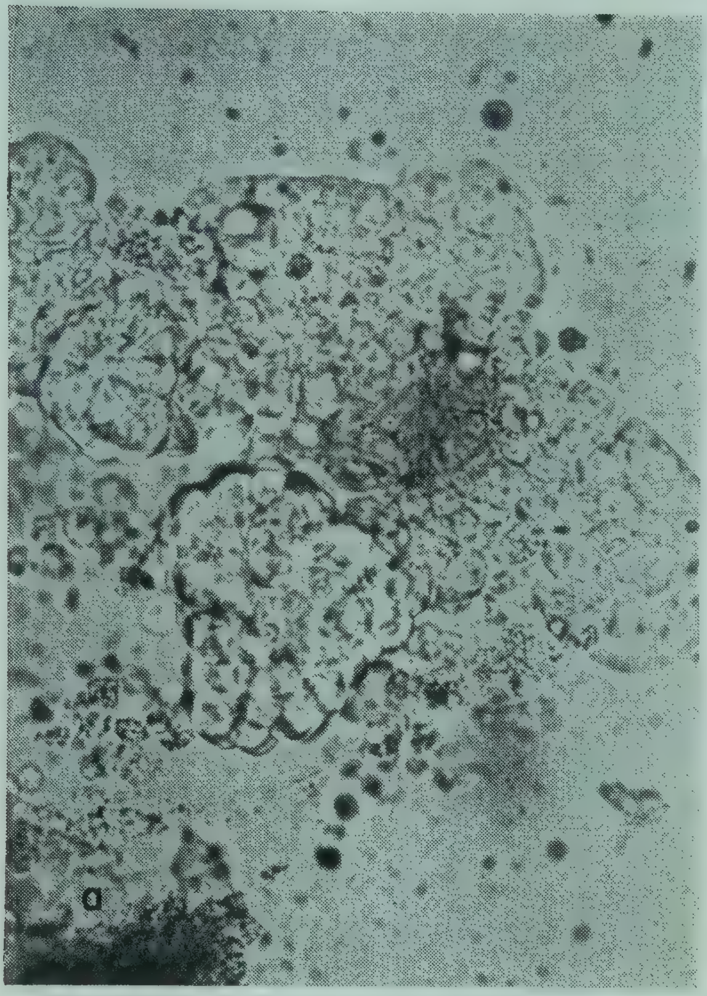


Fig. 42a. Melanophores from a living culture of fish melanoma. 350×.
Fig. 42b. Melanoblast from a similar culture of fish melanoma. 500×.

Their highly pigmented processes remain expanded in the explant or may extend into the medium. Clasmatosis was not observed in these cells.

Carcinomas

Carcinomas are of epithelial origin. In tissue culture their growth resembles that of analogous normal epithelium, although the tumor tissue grows much more rapidly. Rat and mouse carcinomas grow luxuriantly in culture (Fig. 39b). Macrophages appear in the medium first, followed in about 24 hours by growth of compact epithelium in sheets. Their relatively early appearance is in contrast to that of analogous normal epithelial sheets, which usually do not appear before 48 to 72 hours. Many cells within the outgrowing sheet show marked mitotic activity. Frequently the daughter cells are irregular in size and shape both in regard to nuclei and cytoplasm, but later become indistinguishable from the neighboring cells. After a few days, some organization of the epithelium may be observed the type of which depends on the source of the tissue. As proliferation of the epithelium proceeds marked liquefaction of the medium occurs. Some fibrocytes or stroma cells may also be present; these according to most criteria appear to be normal cells.



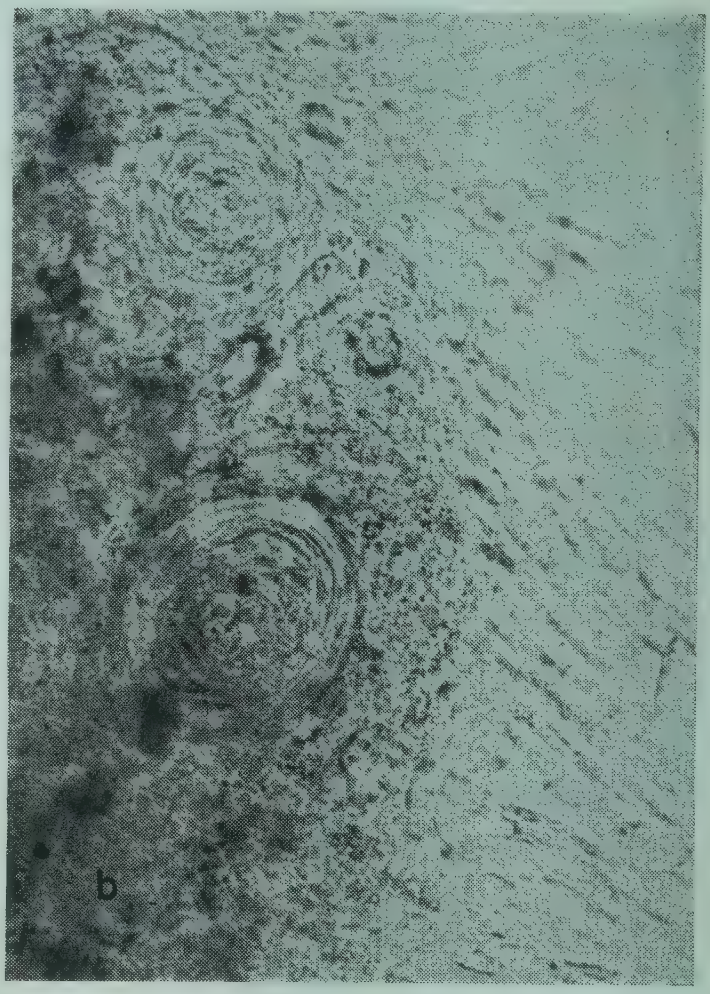


FIG. 43a. Epithelial growth from a primary mammary carcinoma (human). Note formation of acini. 29 days' growth. 300×.

FIG. 43b. Formation of two pearls in living culture of lymph node metastasis of squamous cell carcinoma (human). 14 days' growth. 200×.

We have cultivated many types of human neoplasms in this laboratory. The outgrowth of primary tumors shows the structural characteristics observed in cultures of their normal prototypes. Carcinomas of the mammary and parotid glands develop acini (Fig. 43a) and those of the thyroid develop follicles; squamous-cell carcinomas form "pearls" (Fig. 43b); angioendotheliomas (Fig. 44a) develop blood vessel-like structures, etc., Cameron and Chambers (1937).

Cultures of metastases, especially those obtained from the lymph nodes, proliferate rapidly. The growth usually consists of extensive coherent epithelial sheets interspersed with normal appearing lymphocytes, reticular cells and a few fibrocytes.

Most observations may be made with the medium high powers of the ordinary light microscope. Dark field illumination may also be used and is particularly valuable for observation of mitochondria. Phase contrast microscopy, Firor and Gey (1947), Buchsbaum (1948), Hughes and Swann (1948), is making possible the study of many constituents invisible with the light microscope. (For further details see Chapter XVIII.) Porter, Claude and Fullman (1945), Bang and Gey (1948) have made use of the electron microscope for the study of structures in cells grown in culture. The procedures for this type of examination involve complete desiccation of the tissue.

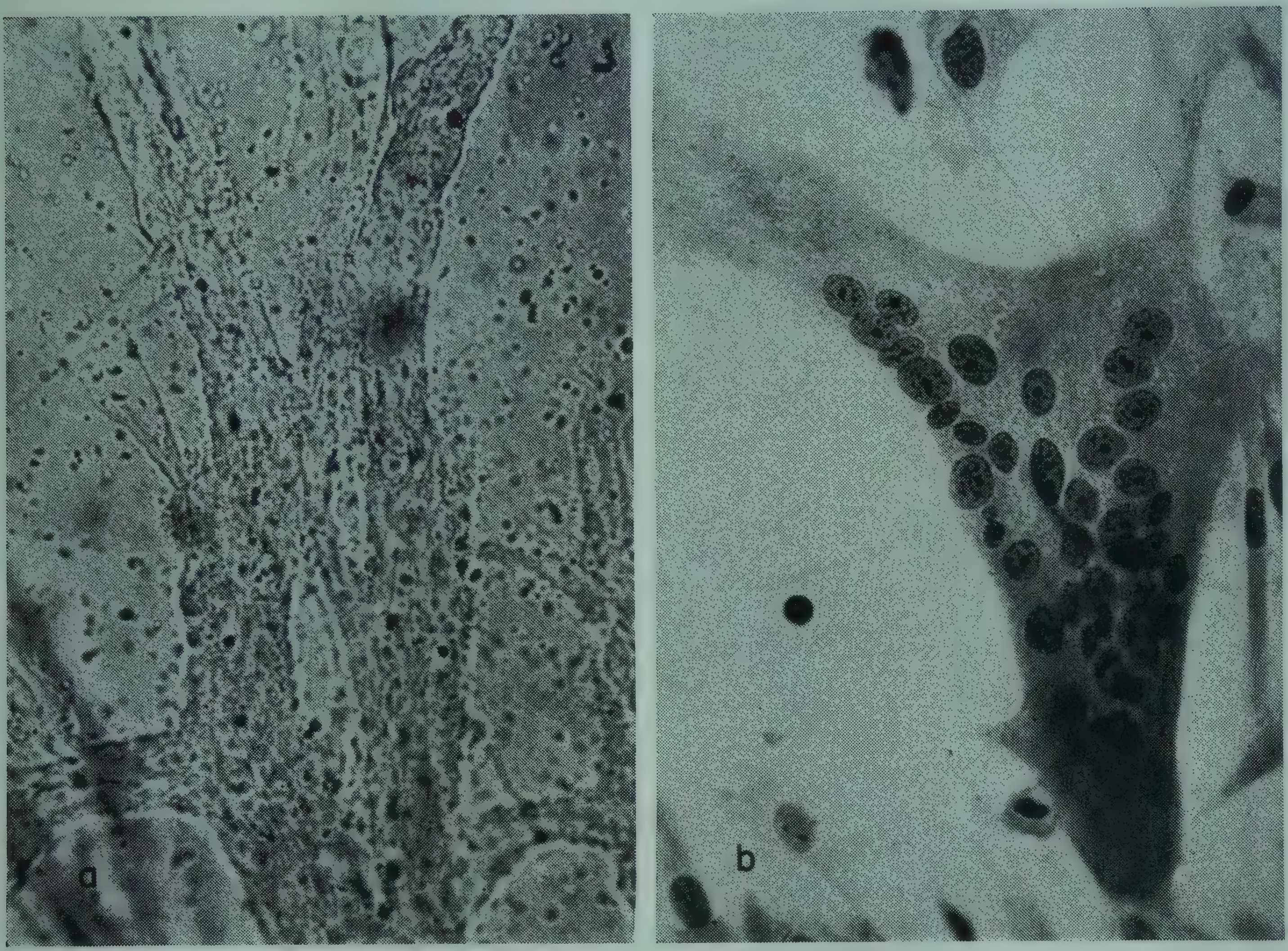


FIG. 44a. Living culture of angioendothelioma, showing tubular nature of anastomosing endothelial branches (human). 32 days' growth. 200×.

FIG. 44b. Multinucleated giant cell from culture of giant-cell tumor of the bone (human). 500×.

Chapter XV

SOME EXPERIMENTAL PROCEDURES

It is a comparatively simple matter to place a small fragment of tissue in a suitable medium and study its behavior under the microscope. Tissues will still grow in spite of a little more or less of this and that in the constitution of the medium. However, it must be realized that variations in the amount of the constituents may affect the nature of the growth and even alter the morphology and physiological activity of the cells. It is quite a different matter when analogous cultures are being prepared for experimental work. In such cases each step of the technique must be exactly controlled.

There are two broad fields of investigation to which tissue culture is well adapted. The first consists of investigations concerned with the study of the living cell, *per se,* such as its nutritional requirements, the interactions of the individual cells with the medium and with each other, and the organization and developmental processes of groups of cells. The second field comprises investigations in which cells and tissues growing in culture are used as test material for a variety of agents to which the cultures are to be exposed.

The coverslip method, permitting high power observation, is the most desirable where cytological studies are to be emphasized while flasks and tubes are more suitable for such investigations as those concerned with nutrition and metabolism. Whatever method is selected there are technical considerations common to all in the preparation of the cultures. For comparative studies the tissue fragments should be as nearly equal in size and thickness as possible (about 1 × 1 × 1 mm.). The constituents of the medium should be accurately measured. This is ensured by using pipettes of uniform diameter and length held perpendicularly when the drops are deposited. The constituents should be thoroughly mixed and evenly spread and, if a clot is desired, allowed to coagulate completely before the culture is moved.

For exploratory experiments, freshly initiated cultures may serve, but for critical work, in which the effect of an introduced agent is to be determined, it is better to use tissues which have been cultured for at least a few weeks, so that they may be in as uniform a condition as possible with respect to the environing medium. The growing cultures should be divided into two parts, one for control, the other for the experiment. Pure strains of cells, such as chick heart fibroblasts as initiated by Carrel (1912) or one of Gey's tumor strains (1936) are excellent for this type of experiment. With slow growing adult tissue it is more difficult to secure analogous cultures. We found, for instance, that sheets of epithelium do not grow from every fragment of adult rat liver. We therefore prepared numerous cultures on double coverslips and, after growth was established, selected cultures for controls and experiment which showed analogous outgrowths. This method is practical for many types of tissue which grow irregularly, such as primary carcinomata.

Account should always be taken of the possibility that the addition of an experimental substance significantly alters the hydrogen ion concentration of the medium. In order to check this a useful procedure, unless specifically contraindicated, is the addition of a small amount of a pH indicator, e.g., phenol red to both the control and experimental media.

KEEPING OF RECORDS

Accurate day by day records should be kept of all work done in the laboratory. A bound record book is recommended. Each worker will probably develop his own system but a typical record as kept in our laboratory will be found at the end of this chapter as a suggestion. Every experiment is given a number and each culture used in the experiment a sub-number.

The cultures should be examined under the microscope frequently, (e.g., at least twice daily) and the observations recorded. For the beginner this cannot be too carefully stressed. He may not be able to evaluate immediately what he sees but once recorded, its significance may be of value in a later analysis.

WHAT TO LOOK FOR

(a) Cultures as a whole

Presence of liquefaction, entire clot or in regions
Extent and density of growth
Changes in pH

(b) Cytological

Deviations of the cells in the experimental cultures from the normal controls. Consider wandering cells, fibrocytes and epithelial cells separately.

1. Do the wandering cells have extended pseudopodia?
2. Are the cells of normal thickness?
3. Is the cytoplasm clear or granular?
4. Does the cytoplasm contain vacuoles: If so, are they large, small, numerous or sparse?
5. Is there a change in shape and size of the nuclei?
6. Are mitotic figures seen and with what frequency?
7. Are multinucleated cells present?
8. Are degenerated cells present?
9. Which type of cell is most affected?
10. Is there a difference in the relative proportions of the cell population?
11. Are there evidences of organized function, e.g., contraction of muscle, secretion of kidney tubules, formation of follicles or acini in glandular explants or outgrowths?

Measurements

Numerous methods have been devised for measuring the rate of growth of cells in tissue cultures and the relative increase in growth area. Ebeling's method (1921) utilizes a *projectoscope.* The image of the culture is thrown on a sheet of paper immediately after preparation of a slide or flask culture. The outline of the culture is drawn as frequently as desired, superimposing subsequent drawings on the original. Precautions should be taken to minimize the harmful effects of the necessary bright light and concomitant heat by using a heat absorbing filter. The various drawn areas are measured by a planimeter and the relative increase calculated. The result is designated the "Growth Index." In all *ocular micrometer* measurements also one dimension only is measured. Various radial measurements are averaged and the relative increase in the size of the fragment calculated. These methods are subject to the same limitation, area only and not depth can be measured. An added complication is the difficulty in determining whether a given cellular sheet is the result of migration or of growth.

Photomicrographs and *camera lucida* drawings may be made at the beginning, during, or at the termination of an experiment. Cunningham and Kirk (1942) report that by direct examination and by use of the camera lucida they were able to obtain quantitative measurements of change in cell size and number and culture area of chick fibroblasts grown on coverslips.

Measurement by *weight* has also been tried but, because small fragments of tissue are injured by undue handling and by subjection to drying, this has been impractical thus far for any large scale experimental work. Moreover, maintenance of sterility is endangered, more or less moisture is bound to be included, and no allowance can be made for the presence of dead cells.

Some investigators have endeavored to measure the degree of the *physiological activity* of a culture. It has been suggested that this may be done in flask cultures by measuring the amount of liquefaction of the medium but no accurate quantitative results thus far have been obtained. Brues, Rathbun and Cohn (1944) determined the *depletion of the medium* and others have adapted the Warburg technique with some success, Gemmill, Gey and Austrian (1940). In all methods used care must be taken that the cells in the cultures being measured are still living.

For *mitotic counts* the most satisfactory way is to sacrifice the cultures and make the counts in fixed and stained preparations. The question of periodicity of mitotic activity must be considered and experiment and control cultures fixed at the same time. The counting may be done in several ways, using a mechanical stage.

1. An absolute count of all stages of mitoses in the entire culture taking into account all levels throughout.
2. A selected number of fields may be counted and averaged.
3. A differential count, noting the number of each stage of mitotic division present.

SAMPLE OF RECORD

CULTIVATION OF RAT LIVER TUMOR INDUCED BY FEEDING DIMETHYLAMINOAZOBENZENE

Exp. No. 2178

Jan. 3	Tumor extirpated. (Rat 23B)		
10:30 A.M.	*Tissue*—Rat liver tumor		
	Method—Double coverslips		
	**Medium*—Fowl plasma	1 drop	2 drops of mixture per culture
	Rat serum	2 drops	
	Chick embr. extr. containing 0.04% P.R.	1 drop	Color—pale pink
	Cultures 1-24		
Jan. 4	Migration of a few wandering cells only.		
9:30 A.M.			
Jan. 5	Cultures 5, 17, 22—Show no growth.		
	All others show active migration of Kupffer cells, and the beginning of epithelial outgrowth.		
	1-7, 9, 11, 14-21 especially active and beginning of slight liquefaction.		

	All washed in Tyrode solution. One drop of fresh medium mixture added as above.
Jan. 6	Epithelial sheets increasing. Cells of sheets are large and flattened and contain numerous granules, a few fat droplets. Mononucleated. Occasional fibroblasts seen, numerous Kupffer cells.
Jan. 7	Culture 22—No growth and medium unduly alkaline. Culture discarded. Cultures 5 and 17 show beginning of epithelial growth. All others show increased growth of epithelium and Kupffer cells with extended pseudopodia. Considerable liquefaction. All washed in Tyrode solution for 10 minutes and 1 drop of fresh medium added.
Jan. 8	Cultures 1, 2, 9, 12, 15 and 20 show growth of tongues of clear epithelium from collecting ducts. Suggestion of development of lumina in some places. This clear hyaline epithelium is in sharp contrast to the heavily granular cells of the sheets of the parenchymatous portion.
Jan. 9	18 slides showing similar outgrowths used for Exp. No. 2190. Others washed and fresh medium added (for stock).

---

* The constituents of the medium are mixed in this order.

EFFECT OF DIMETHYL-*p*-PHENYLENEDIAMINE (DPD) ON RAT LIVER TUMOR INDUCED BY FEEDING DIMETHYLAMINOAZOBENZENE

Exp. No. 2190

Jan. 9
10:00 A.M. 18 cultures from Exp. No. 2178 (showing similar outgrowths of epithelium). Washed in Tyrode solution for 10 minutes.

1-6 controls.

*Control medium—Fowl plasma 1 drop
Rat serum 2 drops
Tyrode solution 1 drop
Chick embr. extr. 1 drop
(0.01% P.R.)
} 1 drop per culture
Color—pale pink

7-18 experimental

Experimental medium—Fowl plasma 1 drop
Rat serum 2 drops
DPD in Tyrode 1 drop
Chick embr. extr. 1 drop
(0.01% P.R.)
} 1 drop per culture
Color—pale pink

	Concentration of DPD Added	Total Concentration of DPD in Medium
7-10	0.0005 *M*	0.0001 *M*
11-14	0.005 *M*	0.001 *M*
15-18	0.025 *M*	0.005 *M*

1- 6 controls—all cells excellent condition.
1:00 P.M. 7-10 No effect—like controls.
11-14 No effect—like controls.

Jan. 9 1:00 P.M.	15-18	Kupffer cells rounded. Glandular epithelium, granular, separating. No effect on ductal epithelium.
4:00 P.M.	7-10	A few Kupffer cells rounded. No effect on other cells.
	11-14	Kupffer cells, rounded, abnormally granular. Marginal glandular cells rounding up. Slight retraction of sheets. Ductal epithelial sheets no effect.
	15-18	Kupffer cells rounded, abnormally granular, probably dead. Glandular epithelium disorganized. Some cells beginning to disintegrate. Some ductal marginal cells rounded.
8:00 P.M.	7-10	Same as at 4 P.M.
	11-14	Increase in retraction of sheets. More rounded epithelial cells.
	15-18	All cells in poor condition.
Jan. 10 9:30 A.M.	1- 6	Excellent condition. Extensive new growth.
	7-10	Kupffer cells active—the few rounded ones recovered. Both types of epithelium growing.
	11-14	Kupffer cells recovering, numerous extended pseudopodia. Separation of grandular cells more pronounced. Ductal epithelium growing. Looks normal.
	15-18	All cells in poor condition. Glandular epithelium disintegrating. Ductal epithelium contains fat droplets, many cells rounded.
6:00 P.M.	1-10	As of this morning.
	11-14	Glandular cells separating and some disintegrating. Ductal cells look normal.
	15-18	All cells dead.
Jan. 11	1-10	Excellent condition.
	11-14	All glandular cells dark, granular, separated, dead. Ductal cells growing, forming cysts.
	15-18	Dead.

Experiment finished.

Conclusion

This experiment shows that the glandular epithelium of rat liver tumor is destroyed by a lower concentration of dimethyl-*p*-phenylenediamine than is the ductal epithelium.

(Cameron, Kopac and Chambers, 1943)

---

* Note that control medium differs from that in 2178 in containing a drop of Tyrode. This does not appreciably affect the growth of the cultures. Its presence is necessary to maintain uniformity, since Tyrode is the solvent for the solute to be tested in the experimental cultures.

Chapter XVI

MICROMANIPULATION OF TISSUE CULTURES

By ROBERT CHAMBERS

The tissue culture technique routinely involves a certain amount of freehand micromanipulation performed in the field of dissecting microscopes, binocular or monocular. By this means surprisingly fine work can be done by the skilled operator and much has been accomplished in cutting parts of the outgrowth from explants, placing capillary tubes containing various solutions in the cultures, etc.

Strictly speaking, the micromanipulative or micrurgical technique refers to the use of precision apparatus for moving microneedles and micropipettes and operating on individual cells in the field of the compound microscope.

The performance of the microoperations under high magnification is made possible by having the cellular tissue suspended from a coverslip which serves as a roof of the microdissecting moist chamber. The shafts of the microneedles pass horizontally over the stage of the microscope through the open end of the chamber so that the tips of the needles, previously bent upward, can be inserted into the tissue from below. Much of the micrurgical work has been done for the purpose of determining physical and chemical properties of protoplasmic constituents, the reaction of protoplasm to injected solutions, etc. (see Protoplasm in recent edition of *Encyclopaedia Britannica*). Some of this has been done on cells in tissue culture and it has been found that tissue cells, with the exception of muscle, are generally too susceptible to survive the effects of intracellular operations, except on a slight scale, for longer than a few seconds or minutes. However, the cytoplasm can be punctured with impunity, filamentous mitochondria can be cut in two and vacuolar membranes ruptured. On the other hand the nucleus, when ever so slightly punctured undergoes death changes. Furthermore, the irritability of myoblasts can be tested by prodding.

Survival experiments of longer duration have been those on the regeneration of parts cut away from sheets of cells, the closing in of holes produced by puncturing sheets and consequent migratory movements of the cells. Much of this sort remains to be investigated.

Except for experiments in which immediate results are to be expected from acute operations, it is necessary that strict sterile conditions be maintained. Proper temperature conditions have also been regarded as essential. For this purpose Péterfi (*Arch. exptl. Zellforsch. Gewebezücht.* **4**, 165 (1927); *Z. wiss. Mikroskop.* **44**, 296 (1927) designed a heating chamber adapted to be fitted with a micrurgical moist chamber of his own design.

A usual type of warm box large enough to hold both manipulator and microscope is also serviceable. This is about the only way to be certain that the culture is being kept at a given temperature. When the heating arrangement is confined to the stage of the microscope there is a risk of undue cooling of the material under observation by the loss of heat from the coverslip to the relatively cooler objective and the microscope as a whole. However, much work can be done at room temperature. Cultures withstand cooling far better than overheating.

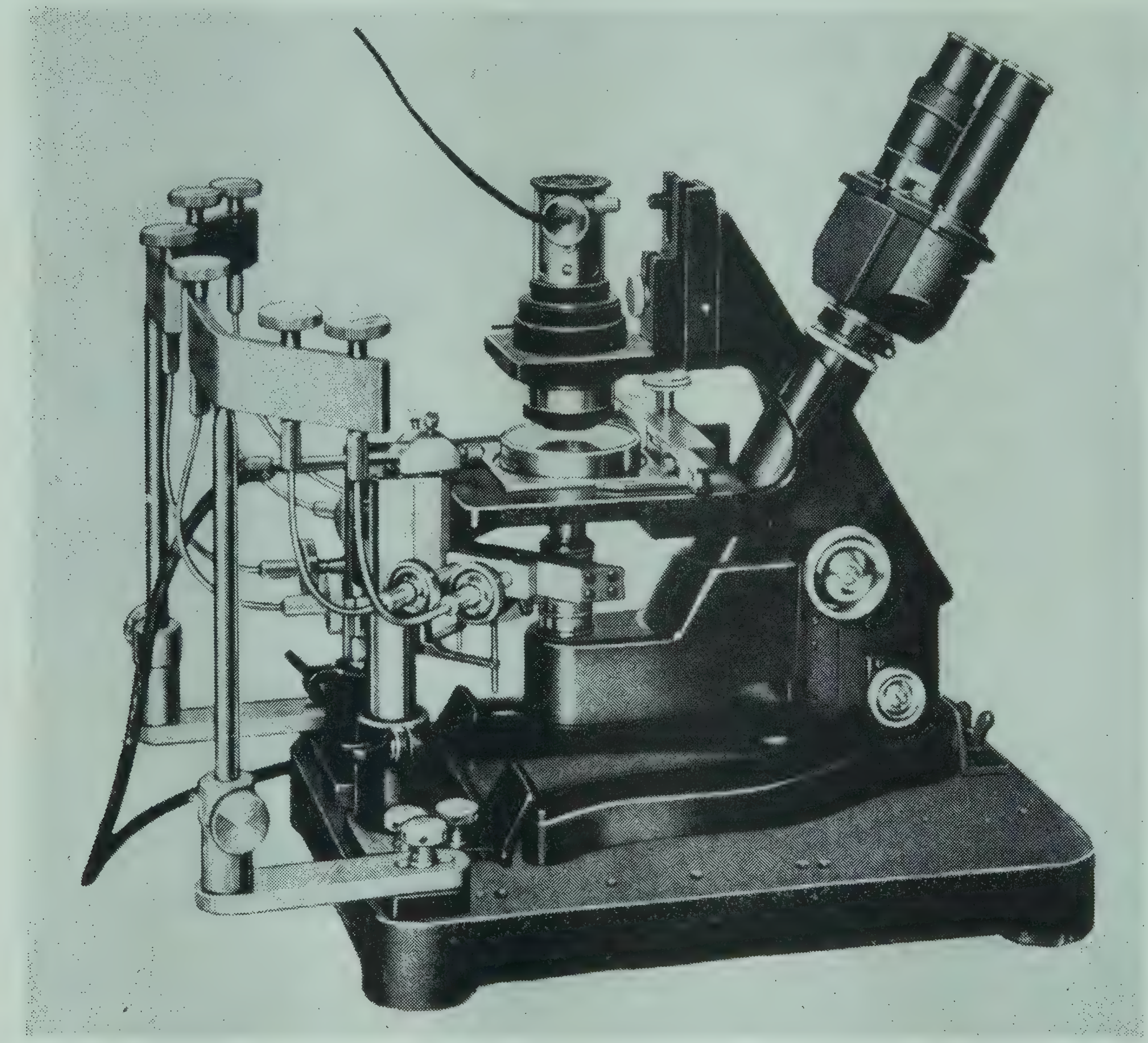
In all microoperative work it is essential to realize that cellular tissue is extremely sensitive. This is reflected in the precautions given for cutting fragments of tissue in the preparation of explants for culturing. Cells have a great tendency to adhere to each other and to their substrate. Dislodging them mechanically often induces irreparable damage.

For microoperations preference has been given to cultures grown in fluid media. Actually, the plasma clot medium offers no serious obstacle, since the clot in the immediate vicinity of the cells to be operated upon becomes readily liquefied by a preliminary moving back and forth of the tip of the microneedle. Subsequent to the operations fresh medium should be added to the culture.

When the clot of the plasma medium is too voluminous or dense, a considerable amount may be digested away with trypsin, leaving only a thin layer of the clot to hold the cells to the coverslip. To do this a 2% solution of trypsin in Ringer's solution may be used. If sterile conditions are desired, the solution is passed through a Berkefeld filter. Place about 10 cc. of the solution in a Petri dish in the incubator and, when warm, float the coverslip with the culture down on the solution, for from 30 to 50 minutes, according to the amount of liquefaction desired. If a sterile solution is used, the action will be a little slower, for a certain amount of the active principle is removed by a Berkefeld filter. When sufficient digestion has taken place, the culture is rinsed several times in Tyrode or Ringer to remove the trypsin and stop further digestion.

A convenient way of handling cultures growing on square or round coverslips (e.g., 24 × 24 mm. diameter) is to mount the coverslip over a hole in a sheet of thin plastic or mica covering the microdissecting moist chamber. When sterile precautions are required a good way is to use perforated metal plates which have been sterilized and between which the coverslip carrying the culture is fastened (Fig. 46).

For this purpose the cultures are grown on "flying coverslips" attached by water capillarity to the undersurface of square mica sheets mounted on Maximow depression slides. When the cultures are ready to be operated upon, the paraffin seal is removed from the slide and the mica sheet with the coverslip attached is lifted off the slide and placed between the two previously sterilized aluminum plates in the center of each of which is a perforation to accommodate the coverslip. The perforation in the lower, thinner plate is slightly less than the dimensions of the coverslip while that of the upper, thicker plate is much larger. The mica sheet thus becomes sandwiched between the two metal plates in such a manner that the rim of the coverslip sits beyond the margin of the hole in the



45. Inverted microscope and micromanipulator (Chambers).

lower plate while the cut-out of the upper plate extends beyond and permits swinging the objectives on the nose piece of the microscope. The completed sandwich is now held together by two marginal clamps and is finally placed on the top of the moist chamber on the stage of the microscope. The set-up is such that the undersurface of the culture is free to be operated upon from below. Several pairs of the aluminum plates cut the size of the dissecting moist chamber should be on hand, previously wrapped and sterilized. After an operation, the sandwich is opened and the coverslip removed with sterile forceps and mounted on a fresh depression slide for further culturing. Figure 46 presents several views of the plates.

Proper illumination is an important factor. For this reason the substage condenser should have a working, focal distance of about 10 mm., the height of the dissecting moist chamber. This can be obtained by using a Leitz triple lens condenser with the top lens removed. If the working distance be less than 10 mm. it is well to have two moist chambers, one for critical work of a height equal to or slightly less than the working distance of the condenser, and the other from 10 to 12 mm. high for ordinary work. This is advisable because it is easier to make needles for the higher chamber.

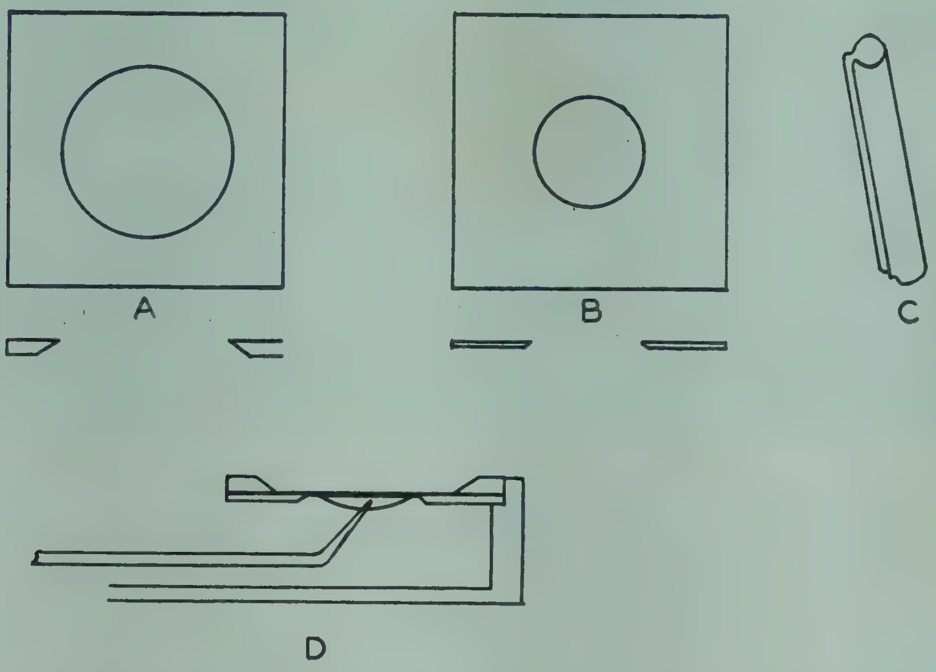


Fig. 46. Diagram of aluminum metal plates for holding coverslips carrying cultures to be operated upon.

The micromanipulator is intended to be used with the mechanical stage of the microscope. The mechanical stage moves the moist chamber. As the cell or tissue to be dissected lies in a drop hanging from the roof of the chamber, the motion imparted by the mechanical stage moves the cells against the microneedle. Indeed, most of the dissection, where a single needle is used, is done by first bringing the needle tip into the cell and then dragging the cell away by means of the mechanical stage.

The horizontal movements of the micromanipulator are used mostly for the purpose of bringing the tip of the needle accurately into a desired spot in the field of the microscope preparatory to the actual operative work.

The manipulator is furnished with needle holders and also micropipette holders consisting of metal tubes with rubber and metal washers at the ends and supplied with screw caps for clamping the shafts of the needles.

The needles are made from either hard or soft glass rods and the pipettes from similar glass tubing. Glass rods or tubes of 3 to 5 mm. in diameter are drawn to capillary dimensions in the flame of a Bunsen burner. The capillary size should be held to an external diameter ranging between 0.5 to 1.0 mm. The microburners can be made from a piece of hard glass tubing bent almost at a right angle and with the upturned end closed except for as minute an aperture as possible which will still retain a flame. This is done by heat-softening the end of the tube and pinching it with forceps. A cutoff hypodermic needle also serves the purpose. Figure 47 illustrates the method of pulling the needle tips over such a flame.

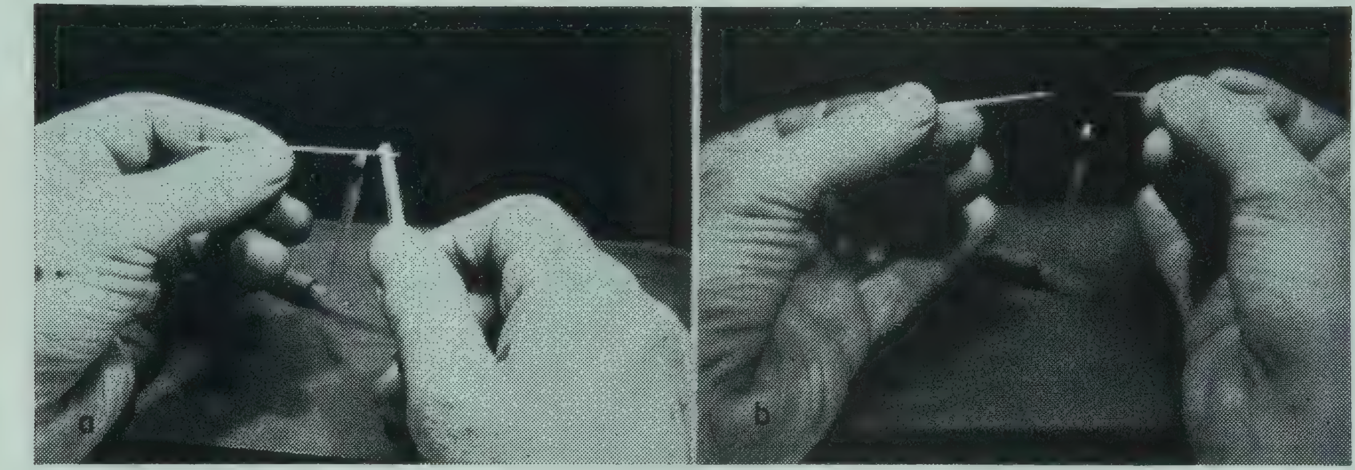


Fig. 47. Two photographs showing the method of making microtips on glass capillaries in the minute flame of a microburner supplied with illuminating gas.

Fig. 47a. Short capillary being held by forceps over microflame preparatory to drawing out a needle point.

Fig. 47b. Similar procedure with a longer capillary held in both hands. A tug at the proper moment moves the heated capillary away from the flame and, simultaneously, draws the glass into two microtips.

To make the needles proceed as follows: 1. In an ordinary burner draw out one end of a glass tube with a capillary of about 0.3 to 0.5 mm. in diameter. 2. Lower the flame of the microburner to the smallest flame possible. Now hold the shank of the tube in the left hand and grasp the capillary at its end either with the thumb and finger of the right hand, or with forceps having flat tips coated with Canada balsam. Bring the capillary over the flame and pull gently till the capillary parts. The hands should remain on the table during the process, and as the capillary parts, lift the glass away from the flame by turning the hands slightly outward. The capillary will separate with a slight tug. If too little heat is used and the pull made too suddenly, the capillary may part with a snap and with a broken tip. If too much heat is used the tip is drawn out into a long hair. 3. Bend the capillary at right angles by heating it just back of the point and pushing up with a dissecting needle. The length of the needle beyond the bend is conditioned by the height of the moist chamber to be used.

A detailed description of the technique is given in McClung's *Handbook of Microscopical Technique,* 1937, Paul B. Hoeber, Inc., New York, a revision of which is in press. A relatively recent account has also been presented in the *J. Roy. Microscop. Soc.* **40**, 113. (1940).

Chapter XVII

HISTOLOGICAL TECHNIQUES

VITAL STAINING

Certain colored compounds added to cultures are helpful in distinguishing details of structure and function. There are two methods by which colored compounds may enter living cells. The first is commonly designated *vital staining*. In this case lipoid and water soluble basic dyes, such as Neutral Red and Janus Green B, diffuse into the cell and stain cytoplasmic granules and mitochondria. These dyes are relatively nontoxic in concentrations of 1-10,000 or below for Neutral Red and 1-100,000 for Janus Green. Coloration of the nucleus is generally an indication of the death of the cell. Brilliant Cresyl Blue 1-50,000 has been used by Grand (1949) for differential staining of cytoplasmic inclusions in cells from Hodgkin's disease nodes. The specific cytoplasmic inclusions color purple to red while other granules color blue.

Methylene Blue on entering living cells is reduced to its colorless leuco compound. If the concentration of the dye is sufficiently low, the cells remain colorless but turn blue on death. A concentration of from 1-20,000 to 1-40,000 is recommended. The vital staining of Methylene Blue is due to the presence of an excess of the dye which tends to be taken up by granules. It has been claimed that Methylene Blue will color a living nucleus. Usually, however, such cells are either moribund or dead.

The second method depends on *physiological activity* of the cell. Colloidal dyes such as Trypan Blue, Lithium Carmine, etc., are ingested by phagocytosis, by cells such as macrophages and leucocytes and to a certain extent by fibrocytes and lymphocytes. Another type of coloration by physiological process is involved when the cells of the secreting convoluted tubule of the kidney transfer an acid dye such as Phenol Red from the external medium into the lumen of the tubule, Chambers and Kempton (1933), Cameron and Chambers (1938). Other acid dyes such as Orange G may be transferred to the lumina and also segregated into vacuoles in the secreting cells while still other dyes such as AzoRubin S form colored vacuoles in the cells only. Coloration depending on physiological activity of the cell is inhibited by cold while the physical process of vital staining is only decelerated but not abolished.

Method of Application

Neutral Red is usually prepared in a 1% solution in distilled water and autoclaved. Higher dilutions are then made in Tyrode solution. The stain may be applied in a variety of ways:

1. It may be added to the medium when the specimen is being prepared or subcultured. There is some danger, however, that the toxicity of the dye even in such dilute concentrations as 1-20,000, will interfere with the normal migration and growth of the cells.
2. A better method is to add a supernatant drop of the dye solution, 1-10,000, to a growing culture and control the degree of staining by examination under the microscope. When the cells are sufficiently stained, the dye solution should be rinsed off with Tyrode solution.
3. The preferred method for cultures on double coverslips is to immerse them in a dilute solution of the stain, 1-10,000 to 1-20,000 in a Columbia staining dish (Fig. 24). Four cultures may be placed in the stain bath and incubated. A culture may be easily removed, examined, and replaced if insufficiently stained. Naturally the thickness of the coagulum is a pertinent factor in the length of time of exposure to the dye. It should also be remembered that the culture medium will absorb some dye and even after removal of the culture from the solution, the staining process will continue.

Overstaining gives a beautiful temporary picture, but the cells will be injured, whereupon they gradually lose the dye and cytolyze. If a nontoxic concentration of Neutral Red is added, the cultures may be maintained as long as the uncolored controls. With renewed medium or successive transplants, mitotic activity will result in diminution of the number of colored granules per cell. Macrophages pick up Neutral Red avidly and, when present, are the first cells to show coloration. The other cells usually stain in the following order of intensity, leucocytes, fibrocytes, epithelium and lymphocytes.

Such dyes as Janus Green B, Janus Black and Methyl Green have a lipoid affinity. Since mitochondria are lipoid in nature these dyes are used to stain them selectively. The mitochondrial stains are more toxic than Neutral Red. In general, a concentration of about 1-100,000 is sufficient. Or immersion of the culture in 10 cc. of Tyrode solution to which has been added 1 to 2 drops of a 0.1% solution for about five minutes provides a suitable degree of staining. Beautiful preparations may be made by mixing Neutral Red and Janus Green. Cells growing on the surface of the coverslip, as they do in fluid medium, or in a thin coagulated medium are excellent for this purpose.

FIXATION AND STAINING

Although the chief value of the tissue culture technique is the opportunity afforded for the study of living cells, it is desirable to compare the living cells with those of similar cultures which have been fixed and stained. Fixation and staining of cultures at the termination of an experiment provides an excellent permanent record. In some cases, it is also advantageous to section the cultures.

Because of the toxic nature of many of the chemical substances used in histological work, it should not be done in the culture room. If a separate room is not available, at least a special table should be reserved for this type of work. A separate cupboard should be provided for storage of the various reagents both to protect them from light and because many of them contain heavy metals and other substances the least trace of which is toxic to living cells. Everything should be carefully prepared, kept clean and clearly labeled. Care and common sense should be used in the disposition of waste materials. Stone jars or granite pails should be used for solids while solvents, stains, etc., should be poured directly into the drain followed by thorough flushing with water. Plugging of the drain with discarded paraffin or celloidin should be avoided.

Columbia staining dishes are convenient for use in the fixation and staining of cultures on small coverslips. The coverslips with the tissue and the clot are immersed in the various solutions and kept in position by the ridges of the dish. Four coverslips such as the 22-mm. squares or circles used in the double coverslip preparations may be accommodated in one dish. A diagram of the position of the coverslips in the dish will make it possible to carry the cultures through the various steps without the necessity for further identification. A cross or other identifying mark may be used to indicate the front of the dish. The worker should make a habit of always placing the culture side of the coverslip toward the front (or back) and of labeling them in a consistent manner so that he is never in doubt as to which side carries the tissue. After dehydration this is sometimes difficult to determine.

It makes for efficient work if the required glassware such as microscopic slides and the Columbia dishes are cleaned beforehand and stored clean and dry ready for use. These staining dishes should be marked and reserved for histological work only and never confused with those used for washing the living cultures.

Tissue cultures grown on coverslips are fixed and stained *in toto*. The coverslips are thin, the cells a delicate layer in the supporting clot which may be rubbed or washed off unless gently handled. It is recommended that cultures grown in flasks or tubes be removed from these containers and transferred to coverslips before the application of histological procedures. Cultures in flasks and tubes may be treated *in situ,* but this is expensive in the case of flasks and makes permanent storage difficult while the nature of the container prevents high power observation.

Almost any accepted histological technique may be adapted to tissue cultures. Those described here are in use in our laboratory. The following is a partial list of the fixatives which are being used for tissue cultures. For a more detailed description regarding the preparation and use of these and other fixatives the reader is referred to the standard textbooks on microscopical technique, such as Cowdry (1943), Lillie (1948).

Some fixatives tend to render the clot opaque. For this reason, especially if the clot is thick, the cultures should be washed in one or two changes of warm Ringer solution for 10 to 20 minutes, then well drained before immersion in the fixative. The time of fixation depends on the size of the explant and the source of the tissue. Embryonic tissue requires a shorter fixation period than does adult tissue.

For cultures grown in *fluid medium* where the cells are in danger of being washed away by immersion in fluids, *vapor fixation* is recommended. The chemical is placed in a depression slide or small Petri dish and the culture inverted over it for from 2 to 5 minutes.

Formaldehyde vapor—from strong formalin—preserves cellular structure, but subsequent staining of mitochondria is difficult.

Iodine vapor—from a crystal of iodine—causes some distortion of the cells and does not fix fat but is excellent for mitochondria which are well preserved and appear dark brown.

Osmic vapor—from freshly prepared 2% solution of osmic acid—is recommended by Lewis and Lewis (1915) and is the most reliable. It produces very little distortion, fixes fat globules, mitochondria and Neutral Red granules.

STAINING

Almost any desired stain may be used. The stainability of the plasma clot must be considered and usually immersion in a dilute solution for a comparatively long period gives best results. The basic dyes such as Hematoxylin and Methylene Blue are primarily nuclear stains, while such acid dyes as Eosin and Acid Fuchsin stain cytoplasm. *Harris' Hematoxylin* colors the plasma clot less than others and is recommended for routine staining of cultures fixed *in toto.* Delafield's hematoxylin also gives good results and is similarly used. Hematoxylin may be used after almost any fixative such as Zenker's, Bouin, Carnoy's, etc.

Fixative	*Special features*	*Time of fixation*	*Subsequent treatment*
Zenker Stock solution 49 parts Glacial acetic 1 part Mix immediately before use	Cytological study Well preserved nuclei and chromatin Increased affinity for acid dyes	½ to 1 hr.	Running tap water overnight Gram's iodine solution for 5 min. Rinse distilled water 5% sodium hyposulfate solution for 2 or 3 min. Several changes of distilled water Is then ready to be stained or may be stored in 80% alcohol
Zenker-formol (Helly) Zenker stock soln. 9 parts Neutral formalin 1 part Mix immediately before use		½ to 1 hr.	
Bouin Saturated (1.2%) aqueous picric acid solution 75 cc. Neutral formalin 25 cc. Glacial acetic acid 5 cc.		½ hr.	80% alcohol, changed frequently until yellow color has disappeared The removal of the picric is hastened by placing the dish under strong light or by the addition of 2% of a saturated solution of lithium carbonate in the alcohol. Wash in running tap water 1 to 2 hrs. Store in 80% alcohol.
Carnoy's solution Absolute alcohol 6 parts Chloroform 3 parts Glacial acetic 1 part	Excellent nuclear fixation Nissl bodies well preserved Considerable shrinkage. Dissolves cell granules and pigments	5 min.	Absolute alcohol 15 min. 95% alcohol 15 min. Store in 70% alcohol until stained
Alcohol-formalin acetic (no previous washing necessary) Distilled water 28 parts 95% alcohol 15 parts Neutral formalin 6 parts Glacial acetic 1 part	Good general fixation Preserves glycogen, fixes pigments	2 hr. to overnight or longer	30%, 50%, 80% alcohol. Store in 80% until stained
Formalin-Ringer (no previous washing necessary) 10% neutral formalin in Ringer May be made in quantity and stored	Good for cellular structure Considerable shrinkage Difficult subsequently to stain mitochondria	1 hr or longer	Running tap water overnight or longer Distilled water ½ hr. Stain or store in 70% alcohol

METHOD FOR ROUTINE STAINING

1. Rinse in distilled water.
2. Immerse in 5% solution of Harris' Hematoxylin, 12 hours.
3. Examine.
   If understained add a few drops more of concentrated dye.
   If overstained dip in 1 drop of concentrated HCl in 10 cc. 80% alcohol followed by a dip in ammonia water.
   For cultures, it is usually wiser to prevent overstaining as this treatment may loosen the clot from the coverslip.
4. Rinse in running tap water, 1 hour.
5. Place in distilled water, at least 1 hour.
6. Dehydrate in 30, 50, 70, 95% and absolute alcohol, about 10 minutes each.
7. To counterstain, 2 or 3 drops of 5% alcoholic Eosin may be added to the 95% alcohol during dehydration. This gives a more even stain than use of a more concentrated solution for a shorter period.
8. Xylol and absolute alcohol equal parts, 10 minutes.
9. Two changes of xylol, at least 15 minutes each.
10. Mount in gum damar or clarite by placing the culture face down on a drop of damar on a slide. Do not use clamps.

Dehydration of a culture in a plasma clot takes much longer than does a section. If the clot becomes cloudy when placed in the xylol, this indicates that water is still present and the culture must be returned to absolute alcohol for further dehydration.

Other stains may be used for special purposes, e.g., *Giemsa,* for white blood cells and for virus inclusions. Fix in Helly's (Zenker-formol) overnight, followed by the usual washing in tap water.

Method for staining with Giemsa

1. Place in 80% alcohol plus a few drops of iodine for 1 to 2 hours. Return to water.
2. Potassium permanganate 1% for 1 minute.
3. Rinse rapidly in distilled water.
4. Oxalic acid 5% for 1 minute.
5. Rinse in four changes of tap water.
6. Rinse quickly in distilled water.
7. *Giemsa* 15 to 20% (use alkaline water, pH 7.0, for dilution by adding sodium carbonate with Phenol Red).
8. Incubate from 4 hours to overnight (38.5°C.).
9. Rinse and leave in distilled water for 15 minutes.

10. Alcohol 80 and 95%, 10 to 15 minutes.
11. Absolute alcohol and xylol, 10 to 15 minutes.
12. Differentiate in clove oil. Rinse well in xylol.
13. Two changes of xylol, 10 to 15 minutes.
14. Mount in Clarite.

Polak's modification of Rio-Hortega's method for mitochondria

1. Fix in 10% formalin in Ringer 1 hour or longer.
2. Wash in tap water several hours or overnight.
3. Place in 1% aqueous uranium acetate for 1 hour at 37°C. in dark (or 12 hours at room temp.).

   Prepare the following solution.

   10% $AgNO_3$, 30 cc.
   5% $NaHCO_3$, 90 cc.

   Add ammonia drop by drop until precipitate is nearly dissolved.
   Distilled water up to 450 cc.
   Store in dark bottle in dark.
4. Place in above solution 15 to 20 seconds.
5. Reduce in 1% formalin in water.
6. Distilled water few seconds.
7. Dehydrate in alcohols.
8. Clear in creosote and toluene or xylene.
9. Mount

Mitochondria are black, the background is yellow to brown.

Slight overimpregnation will blacken the fine processes, especially of the nerve cells.

Spoerri's (1948) tribasic-dye stain for nerve cells

Staining solution:

Cresylecht Violet, 2 g.
Toluidine Blue, 1 g.
Thionin, 0.5 g.
30% Ethyl alcohol, 200 ml.

Filter before using it the first time, but it need not be filtered again on further use. Uncertified dyes sold by Coleman & Bell Co. proved satisfactory.

Spoerri's stain may be used for tissue cultures as follows:

1. Fix in 10% formalin in Ringer, or in Bouin.
2. From 80% alcohol dip the coverslips in the staining solution for 5 to 10 seconds.

3. Dip in acidified distilled water (1 drop of conc. H_2SO_4 in 20 cc. water) 1 second.
4. Rinse in distilled water.
5. Differentiate in 80 and 95% alcohol 1 to 2 seconds.
6. Return to 80% alcohol 1 second.
7. Dip again in staining solution 1 to 2 seconds. (Repeat steps 6 and 7 several times for intense staining).
8. Rinse in distilled water.
9. Dehydrate in 80, 95, and absolute alcohol 2 or 3 minutes each.
10. Absolute alcohol and xylol equal parts 5 minutes.
11. Two changes xylol 10 minutes each.
12. Mount in clarite.

It is necessary to overstain tissue cultures in this stain since much dye will be lost in dehydration. This cannot be hurried because of the plasma clot.

Bodian silver impregnation method

Especially for fine prolongations, such as neurites, as used by Murray and Stout (1947b) for tissue cultures.

1. Preparation:

   Gently spill 0.5 g. protargol into a flask containing 50 cc. of distilled water and allow to dissolve, without shaking, in a dark place. In 3 to 4 hours when the protargol has dissolved, pour the solution into a 10 cc. coplin jar containing 0.5 g. metallic copper.
2. Fixative:

   Bouin or 10% HCHO depending upon the state or type of tissue.
3. Tissue culture method:
   a. After fixation store in 80% alcohol at least 2 weeks, longer does not hurt. (Change alcohol several times while storing.)
   b. Wash (all washings should be in distilled water).
   c. Incubate in freshly made protargol solution for 20 hours.
   d. Wash.
   e. Hydroquinone (1% plus 5 cc. neutral HCHO) for 10 minutes.
   f. Wash.
   g. Gold chloride (1% gold chloride + 3 drops glacial acetic acid) for 1 minute.
   h. Wash.
   i. Oxalic acid (2%) for ½ to 1 minute until purple.
   j. Wash.
   k. Sodium hyposulfite (2%) ½ to 1 minute.
   l. Wash.

m. Dehydrate, clear and mount.

Caution: Use chemically cleaned glassware for solutions and plastic tip forceps. Thoroughly rinse with distilled water each time "wash" is specified.

4. Results:

Nuclei reddish, fine prolongations black.

To preserve Trypan Blue or Neutral Red in fixed preparations, the cultures should be fixed in 10% alkaline formalin (pH 7.6) in Ringer and transferred directly to dioxane, 2 changes of 10 minutes each, then mounted in diaphane dissolved in dioxane. Loosli (1936) recommends fixation in Zenker-formalin the formalin of which has been alkalinized to pH 7.6 with sodium hydroxide. The cultures are then washed and dehydrated as already described.

SECTIONING OF TISSUE CULTURES

The culture is fixed *in toto* and passed through the first steps on the coverslip. Numerous methods are used by different investigators. See Loosli (1936). The following method was developed and used by Grand in our laboratory and has proved to be very satisfactory.

1. Fix in Zenker-formol ½ to 1 hour (other fixatives may be used as desired).
2. Wash in running water 6 hours or overnight.
3. Dehydrate in 50, 70% alcohol, plus a few drops of tincture of iodine for 1 to 2 hours, then 70, 80, 95% alcohol.
4. Absolute alcohol plus a few drops of hematoxylin or strong alcoholic eosin (to help in locating the culture after embedding).
5. Equal parts of absolute alcohol and ether for ½ hour.
6. Embed in celloidin.

   2% celloidin, 25 cc.
   Cedar oil, 75 cc., overnight or longer
7. Chloroform ½ to 1 hour.
8. With a razor blade held at an angle of about 45°, gently remove entire culture, central fragment and clot containing the new growth.

   Place flat on a slide in a drop of chloroform and trim away excess clot as if preparing a culture for transplantation.

   Keep knife and culture moistened with chloroform during removal and trimming.

   Using a section lifter, place trimmed stained fragment in benzol to clear.
9. Transfer to benzol saturated with paraffin for 15 to 20 minutes.

10. Melted paraffin for ½ hour—58 to 60°C. melting point. Two changes.
11. Embed in fresh paraffin and orient.
12. Trim block and cut sections about 5 to 10 μ thick.
13. May be cut parallel to or across the culture. A sharp microtome knife is essential.

Mount and stain in the usual manner.

Chapter XVIII

PHOTOMICROGRAPHY OF TISSUE CULTURES

By C. G. GRAND

THE MICROSCOPE

Any standard make in good condition may be used for photomicrography provided that its various optical parts are in true alignment; these are the eyepiece, objective, substage condenser, iris diaphragm, and mirror. The wide monocular tube is preferable only when very low magnifications are being used, and without eyepiece.

The focusing mechanism must be smooth and have no slippage or lost motion, thereby insuring a stable focus. A multiple nosepiece is convenient for rapid changes of objectives. The mirror should be independent of the moving part of the substage in order that it may remain in a fixed position when shifting the level of the condenser.

When racking up the substage, the uppermost lens of the condenser should come flush with the upper surface of the microscope stage in order to attain the maximum illumination. Care must be taken to see that there are no bright metallic spots on the inner wall of the microscope tube, for these may cause reflection of stray light, which will cause fog marks on the sensitive plate during exposure. Such shining parts can be eliminated by flat black paint.

INVERTED MICROSCOPE

Ordinary microscopes can be inverted for the purpose of observing cells and tissues lying on the bottom of Petri dishes or flasks containing a considerable amount of supernatant fluid. The inverted microscope permits the examination from below of cells in the container with high power objectives having short working distances. This is possible because the objectives are close to the bottom of the container and the illumination is provided from above.

A specially constructed inverted microscope was developed by the writer and is manufactured by E. Leitz (Fig. 45). This instrument is equipped with a multiple objective nose piece mounted beneath a fixed microscope stage. The objective can be focused either by fine or coarse adjustment. The image formed by the objective is transmitted to the eyepiece by means of prisms. A condenser with a long working distance and a lamp attached is arranged above the stage and can be moved vertically by means of a rack and pinion adjustment. The lamp may be substituted by a mirror for use with other illuminators. The binocular attachment seen in the figure may be replaced by a monocular tube for photography. Since the microscope is mounted at an angle, an inclined monocular eyepiece, supplied by Bausch & Lomb Optical Co., Rochester, New York, can be substituted for use with an upright camera.

It is important that the bottom of the glass container should be very thin and its external surface free of distortion. If the surface be uneven this may be corrected by a film of immersion cedar oil covered with a thin coverslip.

Substage Condensers

There are many kinds, depending upon their numerical apertures (N.A.) and focal lengths. The conventional type used for routine laboratory work is the Abbe condenser. The Abbe condenser consists of two highly convex lenses, the combination of which is not corrected either for chromatic or spherical aberration. The three-lens condenser is superior to the Abbe condenser as it is better corrected, both chromatically and spherically, and has a higher numerical aperture.

Aplanatic and Achromatic Condensers

These are highly corrected, both chromatically and spherically, thereby giving color-free range, a great advantage for photomicrography. If possible, this type of condenser should be used in high power work, for it gives high light transmission for short exposures. The N.A. is the same as that of the three-lens condenser. Adding a drop of cedar oil to the top lens and bringing the lens in contact with the undersurface of the object slide will bring the N.A. to 1.40, thus increasing the angle of the illumination cone. In all cases, the substage condenser must be equipped with centering screws and with iris diaphragm. *As the purpose of the condenser is to collect the light from its source and to focus it on the object, it is of the greatest importance that the condenser be properly centered with respect to the objective in order to obtain critical illumination.*

To center the substage condenser, proceed as follows: Close the iris diaphragm as far as possible and rack down the condenser. A sharp outline of the iris will be then seen in the field of the microscope when one is looking through the eyepiece. The image of the diaphragm should be brought into the exact center of the field by the aid of the centering screws. This method is to be used only if the iris is itself in center with respect to the condenser. If not, it should be sent to the microscope manufacturer for centering.

The N.A. of the condenser should never exceed the N.A. of the objective to be used. Since the N.A. of the lower powered objectives is smaller than that of a corrected condenser, the back lens of the objective will be overilluminated. To obviate this the iris diaphragm should be closed at least until the illumination just fills the back lens of the objective. Usually it will be advisable to close it further, depending upon the nature of the specimen. Generally at higher magnifications it can be closed to one half to one-third of the N.A. of the objective. When preparations to be photographed are mounted on thick depression slides, the focus of the condenser may be too short. If so, the top lens of the condenser must be removed in order to increase the focal length and the amount of illumination. The substage is then racked down to bring the cone in focus with the plane of the object. This is also done if very low power objectives are used, in order to form a larger image of the light and to cover the field. The removal of the top lens will not be necessary when a specially constructed long working distance condenser is used.

Dark-field Photomicrography

Dark-field photomicrography requires certain additional precautions. To produce excellent photographs with the dark field, using oil immersion objectives, it is best to select a dark-field condenser which has an N.A. of 1.20. Tissue culture preparations mounted on depression slides cannot be photographed with dark field unless the preparation is remounted on an appropriate flat slide with a drop of Tyrode solution between the coverslip. The edges of the coverslip are sealed with vaseline to prevent evaporation.

The whole preparation mount should not be more than the working distance of the condenser. A drop of immersion oil is placed on the top lens of the condenser, which is racked up until the oil touches the undersurface of the slide. When the objective is brought to focus, care must be taken not to damage the preparation with undue pressure. Whenever the full N.A. of the objective exceeds 1.10, an intermediate objective adapter with an *iris diaphragm* should be used. Its purposes are: (a) to adjust the aperture of the objective so that it is lower than that of the condenser; (b) to increase the contrast so that the object will be sharp against a black background; and (c) to increase definition in thick preparations and avoid glare. Care must be taken that there are no air bubbles in the cedar oil on the condenser. Otherwise the field of view will be turbid or opalescent.

The writer has used with success a dark-field condenser of N.A. 0.8 made by Leitz. It has a focal length of 9.3 mm. and a working distance of 4.5 mm. The advantage of this condenser is that no special adjustment is necessary and the preparations do not have to be disturbed from their original mount, providing the cultures are prepared on thin depression slides which do not exceed 4.5 mm. in thickness. The source of illumination for dark field can be the same as that for bright-field work. This must be so projected with the flat surface of the mirror that the back lens of the condenser is filled by a broad, parallel beam of light.

The photographic plate or film must be of the superspeed type, since the image reaching the plate through the microscope is of necessity weak in light intensity. Slow sensitivity materials necessitate unduly long exposure. To learn the correct exposures tests should be made by the same method as is used for light-field work. It is very important that the glass and the preparation used for dark field be meticulously clean, for dust is an enemy to good dark-field work.

Objectives are of three classes: achromat, fluorite, and apochromat. These in turn are subdivided into three classes: drys, water immersions, and oil immersions. It is necessary to know certain characteristics of an objective, viz., its numerical aperture, depth of focus, working distance, and perfection of its correction.

Numerical Aperture

The resolving power depends upon the N.A. The higher the N.A., the higher the resolving power of an objective will be and the better the definition of minute structures. A numerical aperture of 0.80 will resolve twice the number of lines per millimeter than can be resolved by an N.A. of 0.40. High power objectives in general have higher numerical apertures than low powers, and also a shorter working distance.

Depth of Focus

Depth of focus is the penetration power of the objective. This means the extent to which objects at different levels are simultaneously in focus. The higher the N.A., the shorter the depth of focus.

Working Distance

The actual distance between the front lens of the objective and the object when in focus is the working distance. This decreases as the magnification is increased.

Achromatic Objectives

These are not fully corrected for chromatic and spherical aberrations. The chromatic correction is for only two colors of the spectrum, while the spherical aberration is corrected for one color only. Since the achromatic objectives are chromatically corrected best for yellow-green, the illumination should be used with a yellow-green filter.

Fluorite Objectives

Fluorite objectives (semi-apochromatic) lie between the achromats and the apochromats in the degree of their color correction. If used with yellow-green monochromatic illumination, they will give better resolution than the achromatic objectives and approximate the best apochromats.

Apochromatic Objectives

Apochromatic objectives are superior to achromatic and fluorite objectives with regard to color correction and resolving power. The chromatic correction is for three colors of the spectrum, and spherical correction is for two colors. This is an advantage not only for visual work, but particularly so for photographic purposes. They have high light transmission, thereby permitting short exposure which assures sharpness of image. Apochromatic objectives should be used for photomicrography whenever possible.

Use of Objectives

The achromatic dry objectives have an initial magnification of 1 to 45 ×; the fluorites, 42 to 85 ×; and the apochromats, from 5 to 65 ×. The initial magnification of oil immersion objectives of the first two types ranges from 10 to 114 ×; that of the apochromats, from 60 to 120 ×. The magnification of water immersion objectives is 6 to 90 × for achromats, and 70 × for apochromats. With high power objectives, viz., oil immersions, and if living tissue is used, care must be taken to have the tissue well spread out on a thin coverslip in order to shorten the distance between the preparation and the front lens of the objective, so that it may be brought to focus without danger of breaking the coverslip. When photographs are to be taken of tissues grown by the Maximow method (page 81), the "flying" coverslip should be removed and remounted on a regular depression slide so as to avoid double thickness of coverslips.

The water immersion objectives are preferable when used on objects mounted in watery fluids without coverglass, and give better images than dry or oil immersions. When used with preparations mounted with balsam, errors are introduced that increase with the thickness of the balsam. Distilled water should always be used. The front lens should be grease free; to insure sharpness of image, the diaphragm of the condenser should be shut down as mentioned before. This prevents glaring.

Some objectives are fitted with a correction collar to compensate for the thickness of the coverslip. The rotation adjustments are clockwise for thick coverslips, and counterclockwise for coverslips thinner than normal.

Oculars or Eyepieces

These act only as a magnifying lens for the image formed by the objective. The lower lens of an ocular is termed the field lens and the upper, the eye lens, the former being larger though of a much lower power than the latter. Within the collar of the ocular and in the focus of the eye lens is a circular shelf called "stop," the inner edge of which bounds the field of view. The field lens brings the image formed by the objective to the "stop" and is corrected there. The eye lens magnifies this image. The "stop" should never be shifted from its proper plane. Oculars are of two types: the Huyghenian and the periplane or compensating oculars. The compensating oculars are more highly corrected for color and give a better field of view. In photography they are preferably used with apochromatic objectives.

The oculars used for photography should be of medium magnifications (6-10 ×) and with a maximum flatness of field. The magnification of an image should be obtained with an objective rather than with the ocular.

For observation and photography of living cultures grown in the various types of culture flasks the inverted microscope (E. Leitz) is used (Fig. 45).

Magnification

To determine the magnification of the image a stage micrometer is used with any combination of objective, eyepiece and camera extension. To avoid unnecessary confusion the camera is adjusted so that the ground glass or plate surface is exactly 10 inches from the top of the eyepiece. At this height the marked magnification customarily placed on objectives and eyepieces by the manufacturers can be utilized. These values are then multiplied together to obtain the magnification, e.g., 90 × objective and a 10 × eyepiece gives a visual image of 900 × magnification. If possible a 10 × eyepiece should be standard on the equipment and not changed. The magnification is increased or decreased by changing the objective. The operator should never try to force the magnification but must use an objective that will give him the best image with a sharp definition and the greatest depth of focus. This procedure is especially valuable when photographing cultured cells in which various parts of the same cell are in different planes.

Care of Optical Equipment

To get the best results from any objective it must be handled with great care and not subjected to abuse. A slight trace of dirt or dust will mar the definition. A frequent examination should be made of the front and back lenses of the objectives for dust and fingermarks, especially the front lens. The lenses should be cleaned with lens paper immediately after using and, in case of oil immersion objectives, they must be wiped dry, since oil collects dust. In cleaning oil immersion objectives, the lens paper must have a touch of xylol, which, when rubbed on gently, will remove any oil or dirt that may be on the lens. The lens paper must be kept in a dust-free container and be handled with care, as the dust tends to scratch. The objectives that are not in use should be kept in their respective boxes. The oculars must be kept clean and dust-free, especially the lower lens. The smallest speck will be magnified and may lie in the most important part of the image.

In case there is dust on the lenses of the ocular, the lens on which the dust lies can be discovered by loosening the top lens and rotating it while looking through the ocular. If the dust is on the top lens it will rotate; if on the lower lens, it will remain stationary. The lens found to be dirty should be cleaned with lens paper, not roughly, as the friction will cause static electricity which will attract dust from the air. After cleaning, blow gently to remove any lint that may have come off the lens paper. The condenser and the mirror should be cleaned regularly, especially if the microscope is left standing without a cover. A thin layer of dust on any of the optical parts will absorb some of the light, and lower the intensity of illumination.

Illumination

Incandescent lamps used for critical photography has replaced all others due to the high perfection of electric sources. For critical illumination, the light sources should be of small area with high intensity. At present the arc light is the most intense light source but, due to the amount of care and adjustment required and the intense heat involved, it is unsatisfactory, especially for photographing living tissue cultures.

For regular routine photography, it was found that the low voltage, high amperage, or the ribbon filament type of bulbs, are the most convenient. These bulbs need very little attention other than changing when one is burned out or darkened by extensive use. If used properly with the light filter, it will give a homogeneous light, provided the proper kind of a collector lens is used. Frosted bulbs are unsatisfactory because of dispersion of the light. Daylight is also unsatisfactory because it is difficult to control.

Recently the Western Union Telegraph Co. has developed the "concentrated-arc lamp" which differs from the usual carbon arc light in that it has permanent, fixed electrodes sealed into a glass bulb filled with an inert gas. The cathode side of the lamp is made of zirconium oxide, from which its trade name, "Zirconium lamp," is derived. These lamps produce a very brilliant uniform light source in the form of a spot. The brightness of the Zirconium lamp falls between that of tungsten filament lamps and that of the carbon arc. For photomicrography the 100 watt lamp was found to be the best. It must be remembered that the condensing system for this lamp should be of the highest order so as to produce a beam of light that will fill the back lens of the substage condenser with homogeneous light and without color aberrations. Since the light rays radiate from what is almost a single point the rays will be almost parallel, and produce sharp images of the object for photomicrography. The lamp housing should be supported on a stand so that it can be adjusted to any height or angle. It also is arranged so that the bulb is centered within the collecting lens of the lamp by means of centering screws. The collecting lens should be mounted in a collar and adjusted to focus the light source by turning inwards or outwards. Such a lamp, once adjusted and centered, needs no more attention. It is important that the bulb within the lamp housing is centered with respect to the collecting lens. These lamps give an intense light and can be used for all purposes, from the lowest to the highest magnifications. Irrespective of the type of lamp used, the collecting lens in front of the illuminant should be of a focal length, by which a sharp image of the light source can be focused on the plane of the substage diaphragm of the microscope without placing the lamp at too great a distance from the microscope, since this will weaken the intensity of the illumination. It is desirable to have an iris diaphragm (field diaphragm) mounted in front of the lamp condenser. This diaphragm is used to adjust the size of the field illumination by cutting out superfluous light. It is also important to use a cooling filter in front of the light to prevent overheating of the culture preparation.

The illuminating devices (lamp, filters, etc.) for critical work must be in alignment with the optical axis of the microscope. To insure that it remains thus, it is advisable to fix permanently the microscope and accessory equipment to a rugged table of convenient height and dimensions. If a more portable equipment is desired, these may be mounted on an improvised optical bench consisting of a heavy board that can be carried about the laboratory. To avoid vibrations, thick sponge rubber pads are placed at each corner of the board.

Optical Centering of the Illumination

The most important factor for good results is the centering of the illumination in respect to the optical axis of the microscope. The simplest steps are as follows:

1. Lamp, filter holders and microscope should be aligned and clamped firmly to the baseboard or table.

2. Irrespective of the type of lamp used, the light source must be centered to the collecting lens. To do this, a piece of white paper or ground glass is held against the collecting lens collar, the collecting lens is moved in and out to focus the light source and, if the image on the paper is not exactly in the center, the bulb is raised or lowered. If a lamp is provided with centering screws, these are used to move the bulb so that the light source can be in exact center. The paper or ground glass is removed from the front of the light.

3. Adjust the lamp so that the light hits the center of the plane surface of the microscope mirror.

4. Adjust the collecting lens to center the image of the light source on the surface of the mirror. *Note:* The concave side of the mirror is never used. Only the plane surface will reflect the light without distortion to form an image of the light source and give critical illumination.

5. Close the substage iris diaphragm to its smallest diameter. Focus the opening of the diaphragm using a lower power objective (3×). Center the image of the diaphragm exactly in the center of the microscope field by means of the substage condenser centering screws.

6. Open the substage diaphragm, close the lamp diaphragm and focus this within the field of the microscope. Center the image of the lamp diaphragm by adjusting the mirror. In many cases when the image of the light source has been focused in the field of the microscope, its image may be off center. This is to be corrected by carefully readjusting the light

source by means of the centering screws of the lamp. When the lamp diaphragm is opened the field should be evenly illuminated.

7. Place a stained preparation on the microscope stage and focus with higher power objective (e.g. 30×).

If the illumination is properly centered and the substage condenser adjusted, the image, when focused by raising and lowering with the microscope fine adjustment, will come in and out of focus evenly. However, if the image moves from side to side the optical system is out of alignment and must be corrected.

PHASE CONTRAST MICROSCOPY

The examination or photography of fine details within living cells grown in tissue culture presents a difficult problem even when microscope objectives of highest resolving power are used. The boundaries of thin flattened-out cells are frequently almost invisible because their refractive index does not differ greatly from that of the surrounding medium. The accepted method of accentuating contrast by closing the iris diaphragm of the substage condenser until the diffraction pattern produces a good image does not always demonstrate more cellular detail than when the iris diaphragm is left open. In fact, if the substage diaphragm is closed too far diffraction rings will form, distorting the desired details.

The demonstration within living cells of fine structures such as mitochondria, inclusion bodies, the membranes surrounding fine vacuoles, structure of nuclei and nucleoli, etc., is almost impossible with regular microscopy. All these cellular structures appear in greater detail in phase contrast microscopy. Although the chromosomes are seen with regular transmitted light, they appear darker and with greater detail in phase contrast illumination.

Some of the cellular constituents may be demonstrated by special staining but oftentimes the intracellular structures are distorted. Unstained living cells may be examined or photographed by either dark field or polarized light. These methods also have their limitations; surface layers, rather than internal details are generally revealed.

In order to provide contrast so that structural detail within the living cell may be recognized and photographed, the phase contrast method of microscopy is recommended. Zernike (1935, 1942) was the first to successfully demonstrate this method of illumination. Figure 48 shows the method of image formation by phase contrast accessories. An annular aperture diaphragm is placed in the focal plane of the substage condenser which controls the illumination of the object. The aperture is imaged by the condenser and objective at the rear focal plane. In this plane a phase shifting element, or phase plate, is placed. Light, shown by the solid lines and undeviated by the object structure in passing through the phase altering pattern, acquires a one-quarter wavelength advancement (green light) over that diffracted by the object structure (broken lines). The diffracted light passes through that region of the phase plate not covered by the altering pattern and interferes with the direct undeviated light. The resultant interference affects the two portions of light forming the final image. Altered phase relations in the illuminating rays induced by otherwise invisible elements in the specimen are translated into brightness differences by the phase altering plate. The eyepiece is not shown in this diagram.

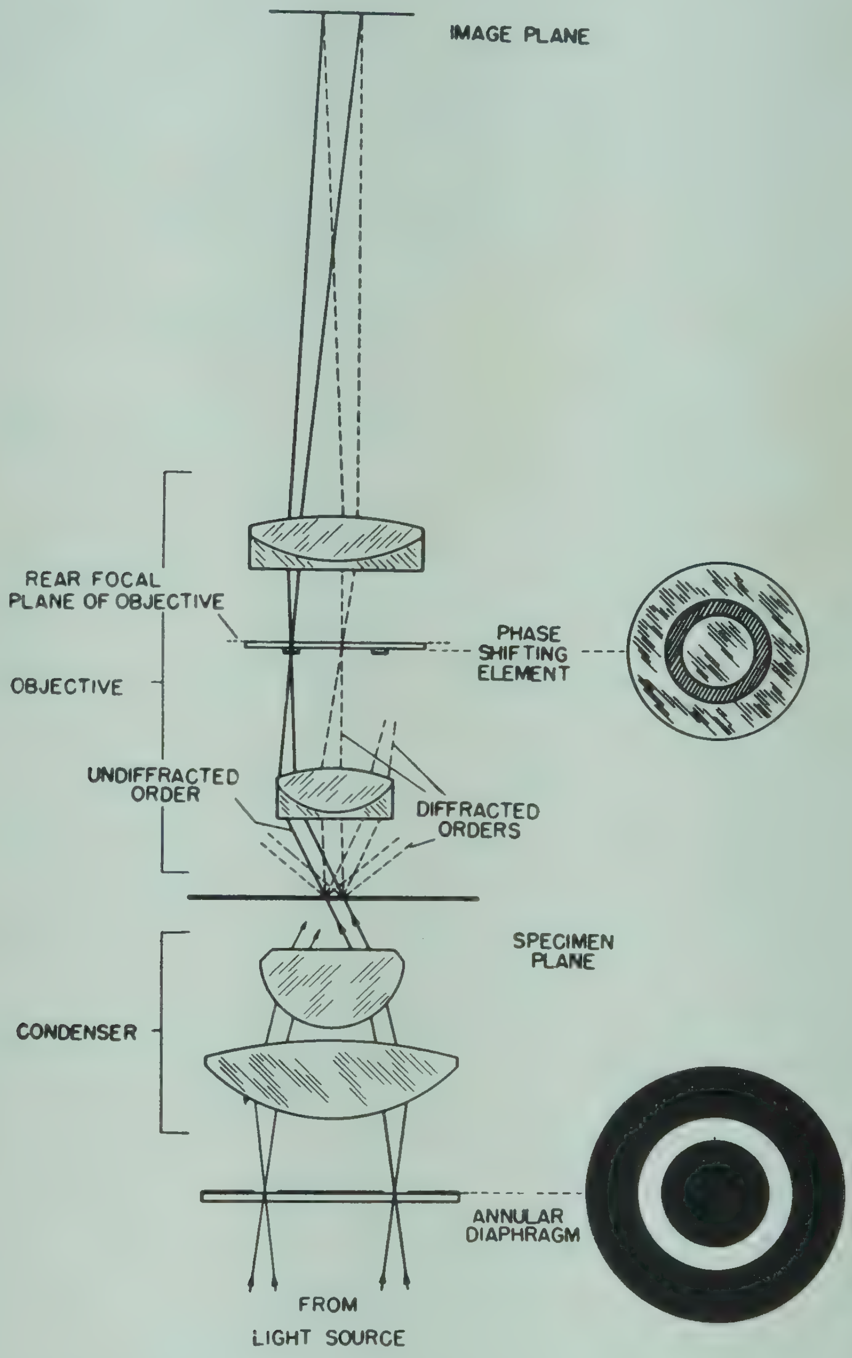


IMAGE PLANE
REAR FOCAL PLANE OF OBJECTIVE
PHASE SHIFTING ELEMENT
OBJECTIVE
UNDIFFRACTED ORDER
DIFFRACTED ORDERS
SPECIMEN PLANE
CONDENSER
ANNULAR DIAPHRAGM
FROM LIGHT SOURCE

Fig. 48. Schematic diagram of phase contrast optics. (Courtesy of Bausch & Lomb Optical Co.).

Many microscrope manufacturers market phase contrast equipment.

It is not necessary to purchase a new microscope, the accessories may be fitted to any standard microscope.

The photography of living cells in tissue culture by phase contrast is comparatively simple if certain precautions are taken. An essential factor is the use of a long working distance condenser if the cultures are mounted in deep depression slides. Bausch & Lomb Optical Co. have developed the individual annular stop long working distance condenser for phase contrast accessories. This condenser has a working distance of from 8 to 10 mm. in air, which is ample for use with cultures under normal conditions. The objectives supplied with this type of condenser have magnifications of 10×, 21×, 43× and 97× (oil immersion). Individual annular stops are available that correspond to the various objectives. Figure 49 shows the basic items needed for all types of work in phase contrast photography. The writer has done extensive work with the above equipment and the results obtained have been very satisfactory.

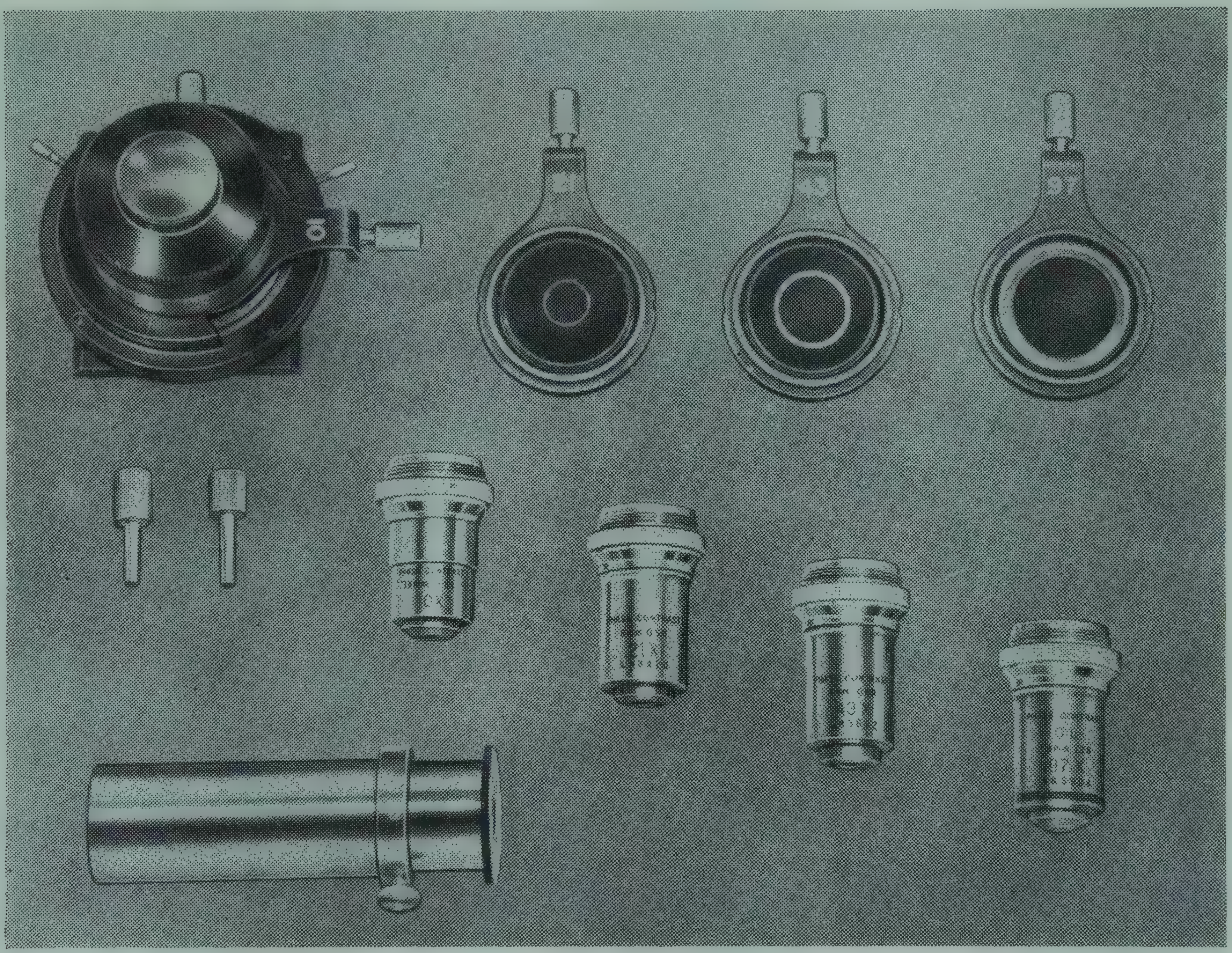


FIG. 49. Long working distance condenser and accessories used for phase contrast microscopy. (Courtesy of Bausch & Lomb Optical Co.)

The regular Maximow depression slide or any other type of concave slide cannot be used for phase contrast microscopy. It is essential that the bottom of the depression slide be optically flat in order to produce perfect images. This type of slide is rather difficult to obtain, however, it may be purchased on special order through Paul Rosenthal, New York.

Cultures on double coverslips are not suitable and should be prepared on single glass coverslips. Mica is not a suitable coverslip for use in phase contrast work.

The steps taken for obtaining proper phase contrast photographs are as follows:

1. Select the proper objective.

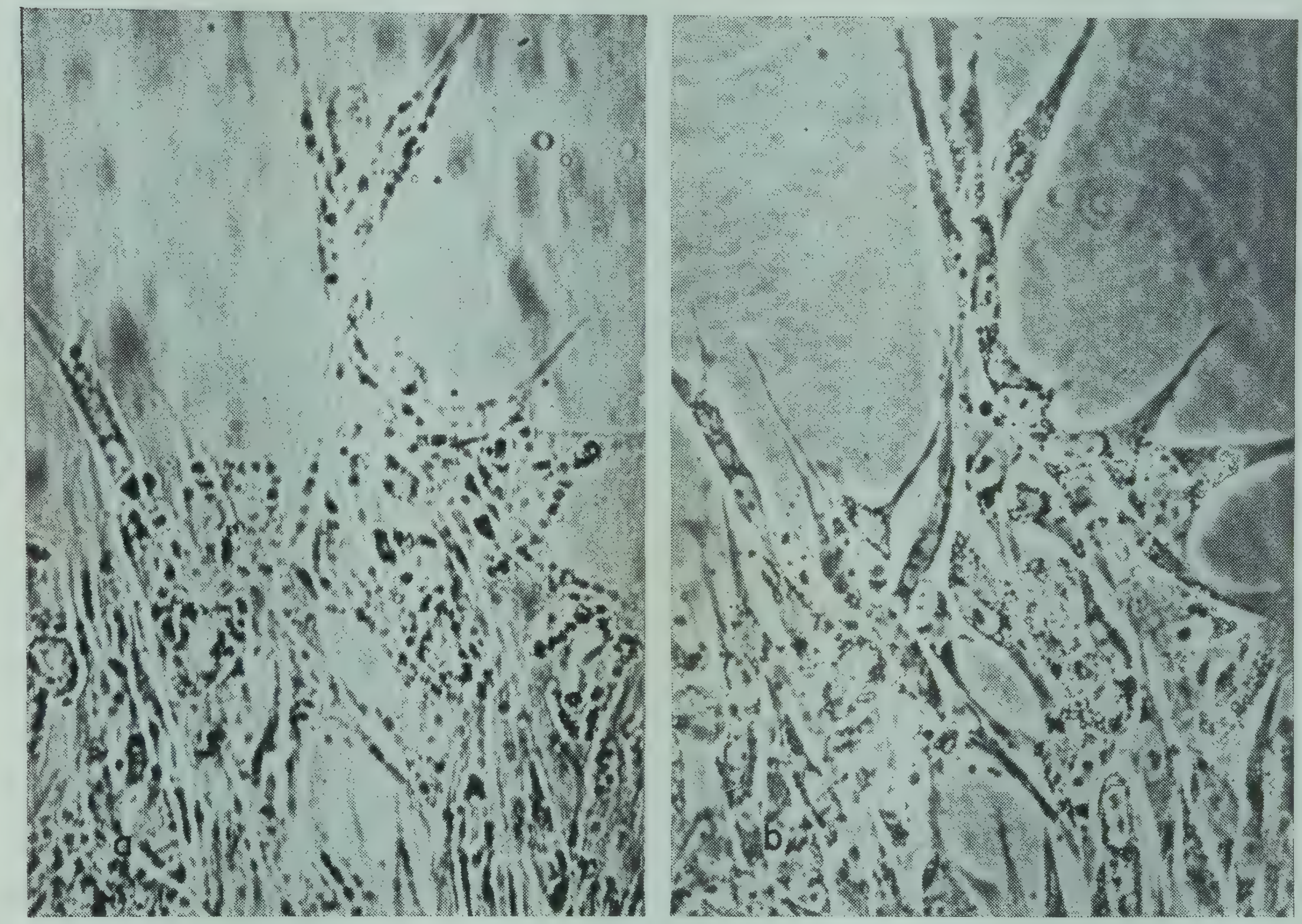


FIG. 50a. Photomicrograph of culture of living fibroblasts with ordinary illumination 210×.

FIG. 50b. Same field taken with phase contrast illumination. 210×.

2. Place the annular stop in the condenser (this stop should correspond to the magnification of the objective).
3. Focus the image with the regular eyepiece.
4. Replace the eyepiece with the auxiliary telescope and center the condenser, annular ring image and the phase altering annulus of the objective.
5. Rack the substage condenser up and down until the whole ring is equally illuminated. It may be necessary to adjust the lamp and microscope mirror to obtain this.

For critical illumination, light is focused on the substage condenser annular ring so as to fill it uniformly with light. Figure 50 shows the comparison between phase contrast and regular microscopy.

There is no advantage in using phase contrast microscopy on preparations which have been stained, cleared and mounted in balsam. However, with vitally stained cultures mounted in an aqueous medium very good images are obtained. The worker may fix his preparations with any desired fixative and, without staining, examine them at his convenience. The coverslip with the culture should be placed face down on a drop of liquid on a regular microscope slide. Figure 51 shows a fixed unstained preparation which demonstrates as much detail as if it were stained.

Regular plates and films may be used for phase contrast photography.

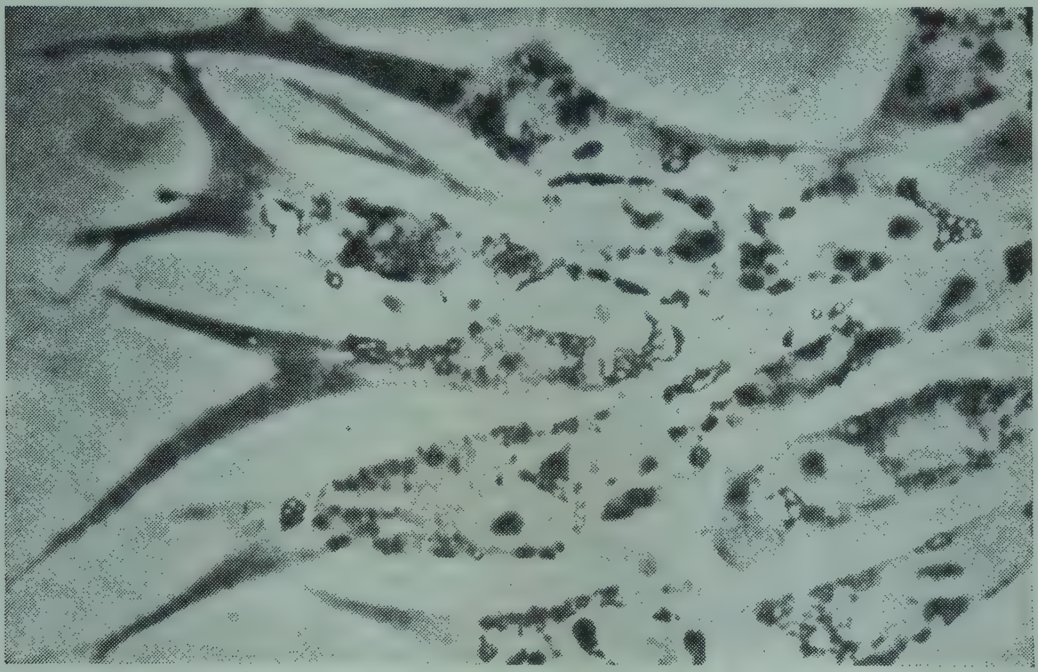


FIG. 51. Phase contrast photomicrograph of fibroblasts fixed in formalin Ringer. Not stained. 1000×.

Since the one-quarter wavelength phase shift produced by the above equipment has been calculated in the green portion of the spectrum, a Wratten B58 color filter should be used.

Color Filters

These are used in photomicrography to increase contrast, thereby bringing out details of structure. In many cases, when photographing tissue cultures which show an extensive but very thin outgrowth of cells, there is not sufficient contrast between the structure of the outgrowth and the background. Color filters are of three types, (a) gelatin dyed with organic dyes, (b) colored glass, or (c) interference film filters. The gelatin filter is quite satisfactory because it may be made to transmit or absorb any part of the spectrum and has a high degree of reproducibility, but it is somewhat unstable. When used in front of a strong light it tends to fade with time, but it can be replaced by a filter with the same characteristics. The glass filter is quite stable to light and heat, and is damaged only by breaking. However, standardization of glass filters has been rather difficult to achieve so that when replacement is necessary the new filter must be recalibrated for filter factors. The interference film filter is a band pass filter having a very high transmission in a narrow band of about 50 Ångström units. They are made for many different transmission bands by most of the optical manufacturers. They suffer no change due to age or exposure to light.

The color filter to be selected must be one that transmits light in that particular part of the spectrum absorbed by the object.

Care should be taken not to increase contrast to such a degree as to destroy the fine details of a structure in high power work. Since most microscope objectives are chromatically corrected and give the highest resolution with yellow-green illumination, a very useful filter is the Wratten B58 green filter. This gives a spectral transmission from 4600 to 6000 Ångström units, which lies between the blue-green and orange-yellow part of the spectrum. This filter can be used not only for unstained preparations but also for preparations stained with hematoxylin and eosin.

For preparations stained with other colors, it will be necessary to have other color filters, the selection of which is determined by the wavelength of the light that the preparations absorb. A useful set of filters for visual study and for photomicrography are the Wratten "M" filters, which consist of nine filters mounted between optical glass. To determine which color filter to use, place different filters, or combinations of filters, in front of the illuminant successively until a filter is found that brings out the proper degree of contrast most satisfactory to the eye.

The required exposure time depends upon two factors: (1) the filter factor which differs with each filter, depending upon the amount of light it absorbs, and (2) the color sensitivity of the plate to be used. Values for the color sensitivity are given by the manufacturers for each type of plate.

The exposure time is best determined either by trial exposures or by multiplying the normal exposure without a filter by the factor of the filter to be used. For instance, if it is found that the normal exposure without filter is two seconds, then, with a filter which has a factor of 14, the correct exposure will be 28 seconds.

Another series of useful filters is the Wratten neutral tint, which are obtainable in sets, transmitting various intensities of light from 50 to 0.01%. Of these, the 50, 25, 10 and 5% are the most useful. They are not color filters, but they may be used in combination with suitable color filters.

Due to a slight yellowish tint, these gelatin neutral filters are not feasible for color photomicrography. Bausch & Lomb Optical Co. have developed four neutral filters that have inconel deposited on glass and are protected by glass covers. These come in different densities of .3, .6, .9 and 1.2 and transmit respectively 50, 25, 12.5 and 6.3% of the illumination. The inconel filters are stable to heat and light and are sufficiently close to a true neutral to make them practicable for color photomicrography. In some cases these neutral filters may be used to modify the intensity of the light without the necessity of closing the iris diaphragm. However, in other cases it is necessary to close the diaphragm even though the neutral filters are used. The procedure will depend upon the nature of the specimen. Still another type of filter is for cooling purposes, which is essential in the photography of living tissues. The colloidal gold glass serves the purpose very well. A liquid filter also may be used. The formula for preparing a serviceable solution is given by Zeiss as follows:

Ammonium ferrous-sulfate (Mohr's salt)	200 g.
Distilled water	1000 cc.
Dilute sulfuric acid (1 part in 3 parts of H_2O)	5 cc.

A glass cell, the opposite faces of which are optically flat, is filled with this solution and placed in front of the illuminant close to the microscope. The width of the glass cell may be from 1 to 3 inches. A stand should be provided to accommodate one or more filters between the illuminant and the microscope. This filter holder must be placed as far away as possible from the illuminant in order to protect the filter from possible damage from heat and from fading as a result of intense light.

CAMERA

For the photomicrography of tissue cultures, especially in liquid media, a vertical camera should be used so that the preparation lies horizontally on the microscope stage. The most convenient type of cameras for this and also for regular routine photomicrography are the reflex or graphic types. These cameras have many advantages:

1. They are transportable.
2. When placed on the proper height table the ground glass image is directly in front of the operator.
3. Without moving, the operator can select and focus the proper field, make necessary illumination adjustments, and instantly shift from observation to photography (by removing the reflex mirror from the path of the light and projecting the image to the plane of the photographic film or plate).
4. The simplicity of operation of this equipment enables the worker to

examine and photograph a large number of preparations with the least effort.

The author has built his own photographic apparatus at very little cost (Fig. 52). It consists of a wooden baseboard with two upright steel rods on which a 3¼ × 4¼ Graflex camera is mounted on a cross member. This cross member, together with the camera, slides up and down on the rods and may be fixed at any height over the microscope with two wing nuts. No lens is used on the camera. Instead, a light trap is placed between the lens board and the microscope. This cuts out extraneous light yet does not allow the camera to be in direct contact with the microscope. The focal plane shutter of the Graflex camera is never used since the

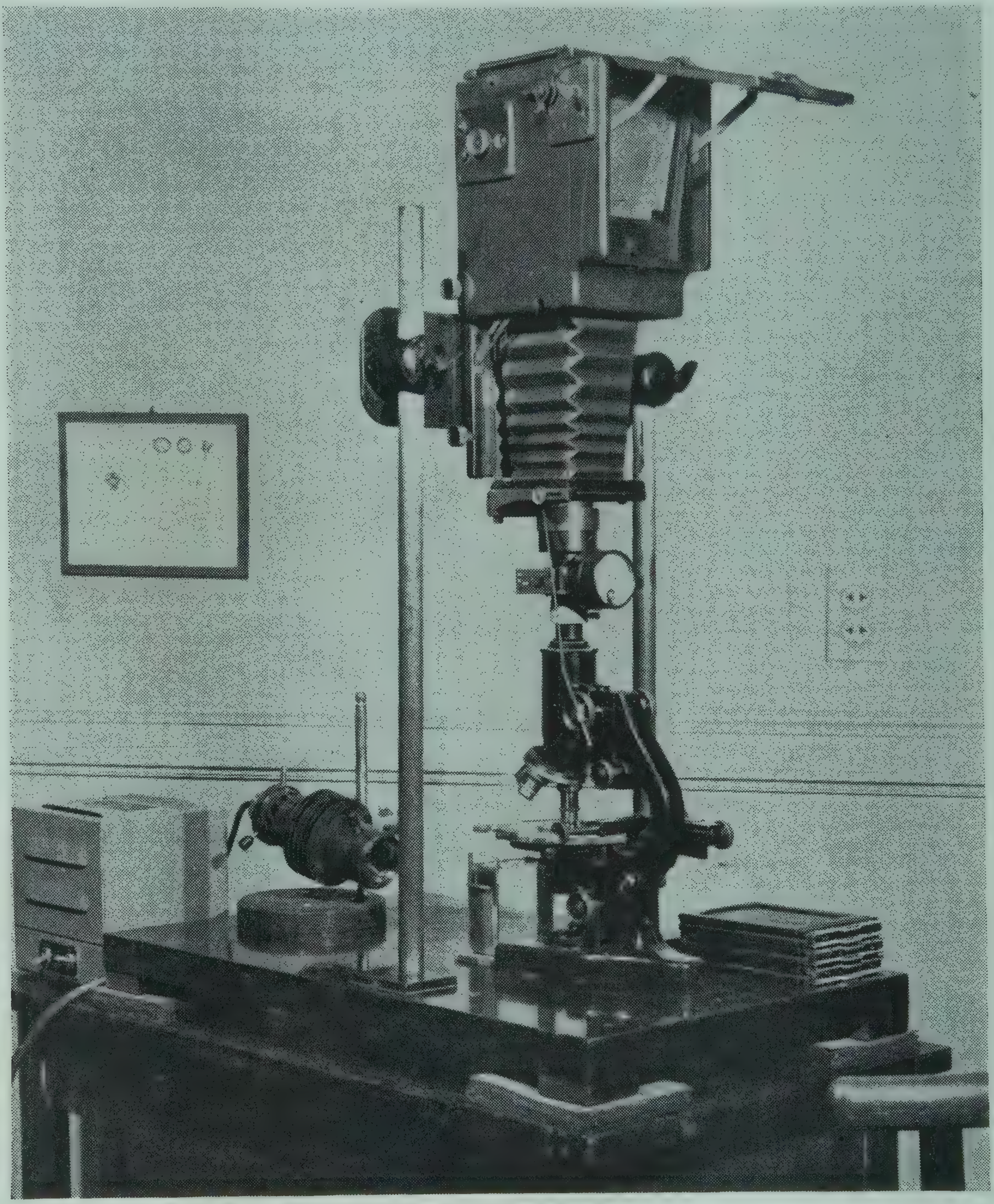


Fig. 52. Graflex camera arranged for photomicrography.

violent jarring causes vibrations that produce fuzzy images. In place of it a compur shutter, mounted between the camera and microscope, is used.

The baseboard is so arranged that the lamp, filters, microscope and camera are mounted as a unit. This unit is mounted on thick sponge rubber to avoid transmission of vibrations. The camera may be removed from the unit and, with a regular photographic lens, used for other types of photography. This equipment has been used for many years and found to be quite satisfactory for all types of photomicrography.

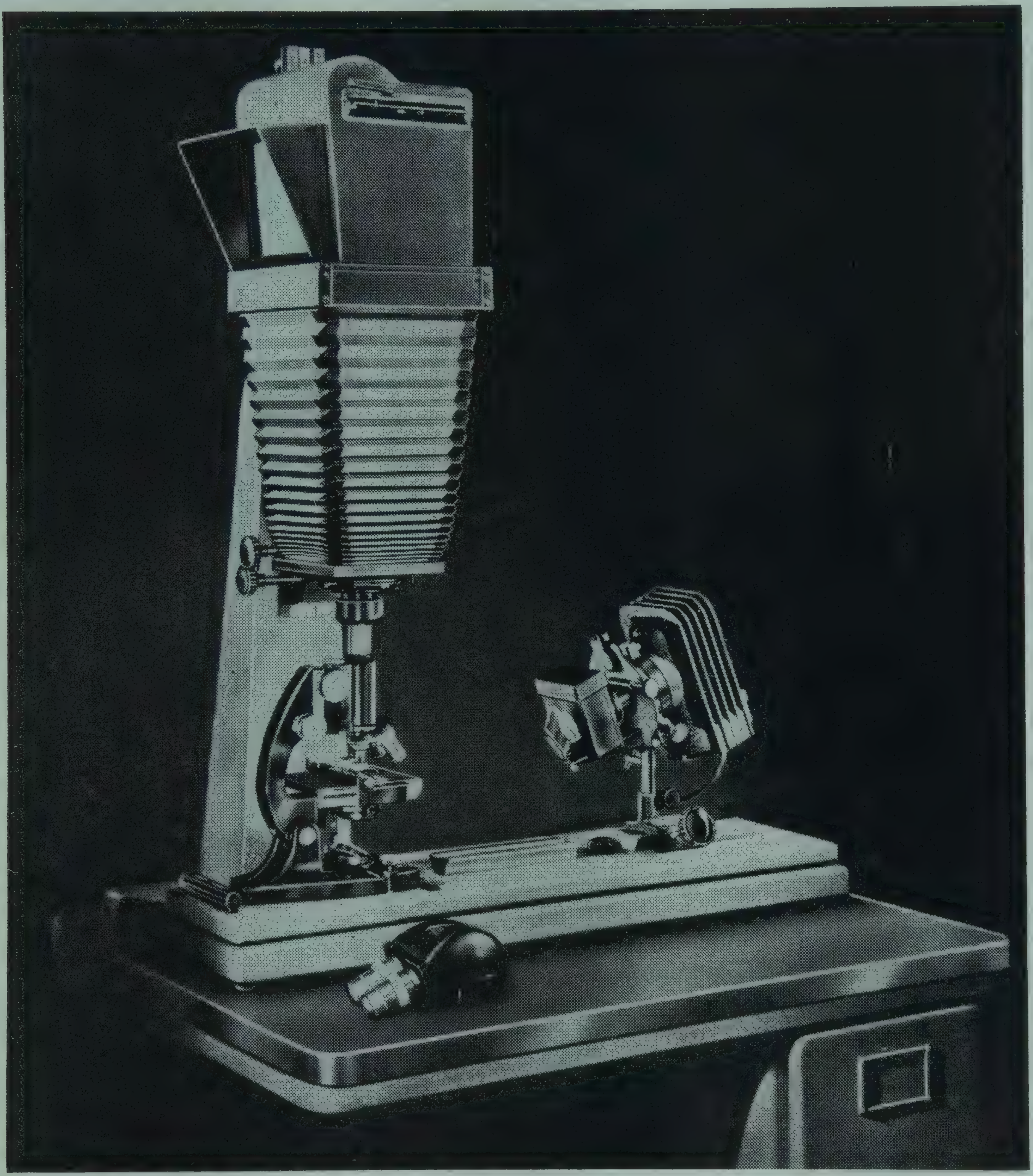


FIG. 53. Bausch & Lomb Model L photomicrographic camera showing microscope in photographic position. Lamp is a special research model.

A more flexible reflex type of a photomicrographic camera is the Model L recently placed on the market by Bausch & Lomb Optical Co. (Fig. 53). It has many advantages; the lamp, filters and microscope are mounted on a platform which slides in and out over another platform on which the camera is mounted. The binocular attachment of the microscope may be used with this equipment for routine examination of tissue cultures. When a photographic record is required the binocular is replaced by the monocular tube, the microscope slid under the camera and the photograph taken without disturbing the optical system. The changeover by this method requires little time and many valuable photographic records are easily obtained.

For use with any of the above cameras the author prefers plates or films of the 3¼ × 4¼ size. They are easy to handle and process with minimum cost and may be procured in various types of emulsions from most photographic dealers. If larger prints are required the entire negative or a portion of it may be enlarged to any size. Color films may be used as lantern slides with very little trimming.

There are certain advantages in the use of 35-mm. cameras. They are economical to operate and use standard 35-mm. film in black and white and in color. It is well known that the resolving power or ability to differentiate details in structure depends on the objective of the microscope, and with the use of the 35-mm. film this detail can be recorded. The distance between the eyepiece and film is so short that ample intensity of light can be obtained with a low wattage and shorter exposure. Such cameras are made to fit into the tube of a standard microscope and because of their light weight no special fastening devices are needed. Because the operator is able to take many exposures (18-36) with one loading of film, it is of great help when taking photographs of an experiment at time intervals or recording a large number of cultures. These cameras are far simpler than larger ones and when used with care are capable of producing photographs of very high quality. The fine grain films and fine grain developers of today permit enlargements to be made which are difficult to distinguish from contact prints made from larger negatives.

To fit a 35-mm. camera to the microscope, the Micro Ibso attachment manufactured by Leitz can be used. This attachment is equipped with a viewing and focusing side telescope with the regular eyepiece connected by a collar. A compur shutter is used for making the exposures. Any model 35-mm. camera, e.g., Leica, Argus, Kodak 35, etc., may be adapted to the Micro Ibso attachment. However, care should be taken that the focus on the film plane coincides with the focus through the side observation telescope. The side telescope is equipped with an adjustable eye lens and cross lines. To obtain the proper focus on the film the side telescope is adjusted to the eye of the operator by looking through and focusing the fine cross lines. These lines should appear sharp and without halo. When this is accomplished the image should be sharp on the plane of the film. Since the light is partially transmitted by means of a prism to the side telescope it is necessary to increase the exposure time accordingly. By pressing the prism cable release the prism, which reflects the light, is moved from the field so that all light reaches the film.

A reflex type attachment using the Argus 35-mm. camera has been developed by Erb & Gray of Los Angeles, under the trade name "Visicam." The camera is provided with a 2-inch ground-glass viewing screen with a black line frame for delineating the 35-mm. field. No other support is necessary in attaching the camera to the microscope tube.

Other reflex 35-mm. cameras, e.g., Kine Exacta, Praktiflex, etc., may be adapted to photomicrography by special means designed by the individual investigator. It makes little difference what type of camera is used but it must be remembered a smooth working shutter is necessary. The timing should be from 1 to 1/100 of a second, including time and bulb settings.

Plates and Films

There is a large variety of sensitive emulsions on the market which give the worker the opportunity to select any kind of material for his specific needs. The choice ranges from the slow process to superspeed panchromatic emulsions, including the special spectroscopic plates and color films. Whether to use glass plates or cut film is left to the worker's discretion. Both have their advantages and disadvantages. Special types of emulsion for photomicrography are obtained only on glass plates so that the danger of breakage is great and they are bulky to store. On the other hand, cut films are unbreakable and easily stored but must be handled carefully to avoid scratches or damage to the emulsion and base surfaces.

Plates and films with an antihalation backing should be used for photomicrography. This is important as halation causes the loss of resolving power of the emulsion. The resolving power of photographic material is conditioned by the inherent contrast, light scattering power and the light absorption of the emulsion. This is expressed in the number of lines per millimeter that can be distinguished separately in a photographic image. However, maximum resolution does not only depend on resolving power of the negative material but also depends on resolving power of the microscope objective and the method used in processing the negative. For photographing fine cellular detail, e.g., granules, chromosomes, vacuolar membranes, etc., high resolving power objectives and emulsions are best.

The beginner will do well to standardize on two or three types of plates or films and familiarize himself thoroughly with their characteristics, especially latitude of exposure, contrast, color sensitivity and resolving power of the emulsion. It should be noted that contrast partly depends on the speed of the emulsion. Slow emulsions give higher contrast than fast ones. Plates or films should be able to produce negatives of various contrasts (high, medium, low) when developed under the specific conditions called for by the manufacturer.

Two types of plates have been developed by Eastman Kodak Co. for photomicrographic work, the Wratten "M" Panchromatic plate and the Wratten "Metalographic" Orthochromatic plate. These plates have a medium speed, fine grain, high resolving power and some of the qualities of regular process plates; and produce negatives of a wide range of contrasts by appropriate development. The Wratten "M" plates are sensitive throughout the whole visual spectrum and consequently have high color sensitivity. With red-colored filters they are used for photographing stained preparations. For routine photography of living or stained tissue cultures the Wratten "Metalographic" plate is the most satisfactory. This plate has an unusually high sensitivity in that region of the spectrum transmitted by the green filter and gives the best definition when used in conjunction with achromatic objectives.

When photographing living unstained cultures in which maximum contrast is desired, color sensitivity is unnecessary in the plate and a contrast emulsion as found in a process plate or film is appropriate. This type of emulsion, which has a very high resolving power, is sensitive only in the violet and blue region of the spectrum. Resolution is not only dependent upon the aperture of the objective but it also increases as the wavelength of the light is decreased. The use of a blue-violet filter (C) with process plates or films will produce brilliant images, and be almost as fast as medium speed panchromatic materials.

Cut films may be developed having the same characteristics as those obtained in plates by the use of proper color filters and processing methods.

Thirty-five millimeter cameras have become very popular and film manufacturers have developed extra fine grain film to be used with them. As is true for the larger size, there is quite a selection of 35-mm. films for the worker to choose from, but he should restrict himself to those which have fine grain and give good contrast, color sensitivity and speed. It must be remembered that the faster the film, the coarser is the grain, even when an extra fine grain developer is used.

The superspeed, panchromatic 35-mm. films are most satisfactory for instantaneous exposures since they are the fastest and most color sensitive of films yet produced. These films give excellent color rendering and allow the use of orange or dark-red filters. They also give good color correction for stained preparations. All these films can be purchased in bulk from which suitable lengths can be cut. Or, in the event that the investigator wishes to purchase film ready for immediate use, he will do well to purchase it wound on a spool containing 18 or 36 exposures, already cut, for daylight loading.

Color Films

Almost any of the commercial color processes can be applied to microscopical work. Color photomicrographs should only be taken of stained tissue cultures since unstained preparations will not show any more than when taken in black and white. The simplicity of Kodak Ektachrome, Kodachrome Type B and Ansco color films make them highly desirable for this type of photography. All of these films are used with tungsten illumination of proper "color temperature" (degree of whiteness of the lamp as specified by a special scale of temperature and denoted as degrees Kelvin or "°K").

The "°K" depends upon the voltage applied across the lamp. To measure the color temperature of the illumination the Eastman Color Temperature Meter is used. If the °K of the lamp is found to be different from that for which the film is balanced, it is necessary to make some compensation, either by changing the voltage across the lamp or using color temperature compensating filters in front of the illumination. In either case a voltage regulator should be used with the lamp to maintain constant °K.

Ektachrome and Ansco color films may be processed by the worker himself but the manufacturer's instructions must be followed carefully to ensure good results.

The microphotometer is a valuable adjunct in color photography and, after the first test for exposure time, the operator can take as many color photographs at any time and send them to the manufacturer for processing.

Exposure

No definite set rules can be laid down for the proper time of exposure. This depends upon the magnification, illumination, light filter and sensitivity of the plate or film. The exposure time varies as the square of the magnification, e.g., an increase in magnification of three times requires nine times the exposure. This is true regardless of the objective, eyepiece or bellows length for obtaining the magnification.

Photoelectric exposure meters for photomicrography both in black and white and color are of great value. They enable the worker to obtain correct exposed negatives without uncertainty, especially when a large number of preparations are being photographed. Developing the plates or films by the time and temperature method ensure the negatives of having the same density.

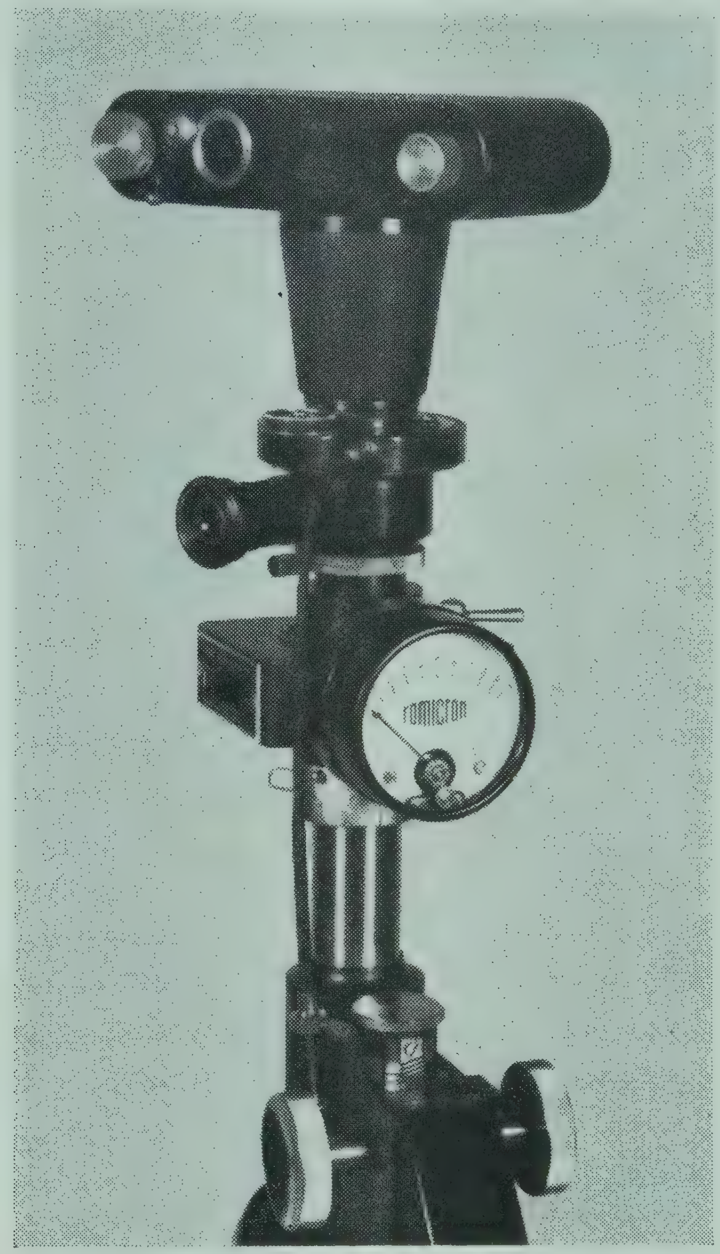


FIG. 54. Photoelectric cell mounted between microscope and camera. Micro Ibso attachment with the Mifilmca 35-mm. camera attached.

Many types of photoelectric exposure meters have been developed by various investigators for their own needs. The writer has developed a microphotometer (Fig. 54), marketed by Paul Rosenthal, New York. This instrument can be adapted to any standard microscope and type of camera. It is placed between the microscope and camera without the need of dismantling any part of the equipment to obtain a reading of the light intensity. A microammeter serves as an indicating instrument with an arbitrary scale of ten divisions with their subdivisions. For a reading, the selenium photocell is placed in the path of rays by operating a lever mounted on the housing, at the same time not disturbing any part of the optical system. A range switch mounted on the side of the microphotometer housing permits a change of sensitivity, and a female plug in the same place allows an amplifier or galvanometer to be attached to increase the scope of the instrument.

The instrument is calibrated by having an adequate light source adjusted to such an extent that a convenient scale reading may be had without the use of any color filter in front of the illumination. A sensitive plate or film is placed in the camera and the cover for the plate holder pulled out part way. Exposures are made of any arbitrary time, the cover is then partly moved out again and the same exposure repeated until five or six exposures have been made on the same plate or film. In this manner, different parts of the same plate will have been given exposures of successively decreasing lengths and, so exposed and developed, will give a range from which the correct exposure can be selected. Having determined the best exposure time for a certain reading, it is then an easy matter to correlate values for the entire scale. All measurements should be calculated with white light and the result multiplied by the proper color filter factor. The best procedure is to measure the average amount of illumination over the entire field. However, an eyepiece with an iris diaphragm, supplied with the instrument, may be used for measurements of small areas within the field, e.g., measuring absorption and transmission of light through a specific section of a stained cell. Densities of plates and films are also determined by this method.

Darkroom

A plate or film should be handled with the greatest care. A great enemy of sensitive material is fogging, which is caused by stray light in darkrooms, too strong a safelight, or wrong safelight screens. The darkroom must be lightproof. To test this, the worker should remain in the room for about ten minutes in total darkness at which time he will become aware of any leaks of light. If the light comes through cracks around the door, they can be eliminated by using the rubber weather stripping generally used for windows. This is tacked around the door so that the greatest width of the stripping will cover the cracks. However, a darkroom should have a light-trap doorway allowing the worker to walk in and out without admitting stray light.

The use of a strong or direct safelight is a mistake that is not generally realized. A darkroom safelight to be safe must be one which gives only reflected light not strong enough to cause fogging of a plate 3 feet away in 3 minutes.

All panchromatic and color materials should always be handled in total darkness since they are sensitive to all colors.

It is not necessary to have the darkroom walls painted black, since the light used in the room is filtered through a safelight and is safe because the white walls reflect only this light. Moreover, white or cream color walls remove the dreariness of the usual darkrooms.

A deep sink with an overflow pipe is essential. Clean water must be circulating constantly for washing plates and films. The darkroom should be kept clean, and splashing of the developing solutions avoided, for these may dry into particles of dust which are carried into the air and may fall on sensitive materials. This is a very usual fault causing stains and pin-point spots on otherwise perfect plates.

There is also danger of fogging sensitive materials if the darkroom is close to a chemical laboratory where there is a chance of chemical fumes seeping into the darkroom. The writer stresses this point, because even unexposed sensitive materials stored in such a darkroom may be ruined in this way.

Developing

The worker should depend upon technical information and formulas published or marketed by various makers of sensitized materials. Through great effort and experimentation these firms have developed special formulas and methods for processing a specific type of emulsion and, if their instructions are followed carefully, good results can be obtained.

Developing tanks are always used for processing negatives, and time-gamma-temperature methods for developing. The time-gamma-temperature method is based on a given developer that will develop a specific type negative material to a known density at a known temperature. A negative may be processed in trays and inspected, but this is not satisfactory due to the constant handling of the negative and exposure to the air, which causes fogging. By the inspection method there is always a tendency for the worker to remove the negative from the developer before full development of the detailed image. When using processing tanks the temperature of the solutions remains more or less constant. Handling is kept at a minimum when plate or film developing racks are employed.

Gamma (γ) has been defined as the numerical factor or measure of the degree of contrast to which a photographic emulsion is developed. A direct relation exists between the time of development and the degree of contrast produced in the negative, the gamma increasing in value as the developing time is lengthened. In some cases of prolonged development the gamma value reaches a maximum and then diminishes slightly due to the proportionately greater fog densities. The degree of contrast obtained in a developed negative is influenced by several factors:

1. Inherent emulsion contrast.
2. Type of developer used.
3. Developing time.
4. Temperature of the developer.
5. The amount of agitation of the plate in the solution.

Time-gamma-temperature developing charts are supplied with each batch of photographic materials and if not, technical information pertaining to a particular emulsion may be obtained from the Photo-Lab-Index. The accumulation of information on photographic materials found in the Photo-Lab-Index is of great assistance to the worker.

The relationship between gamma and graininess of a developed emulsion is direct with many types of emulsions. The graininess increases as the emulsion is developed to progressively higher gammas. Therefore, care should be taken to use fine grain developing methods to avoid development to excessive gamma values. Development to a suitable gamma is of critical importance, for overdeveloping may produce undesirable blocking of details, underdevelopment, a lack of detail. In the time-gamma-temperature developing procedure it is essential that the negative should have received a correct exposure so that, when developed at a certain time and temperature, it will produce a negative showing fine details within the structure of the cells. The gamma should have a range of from 1.2 to no more than 2. In many instances negatives that have been over- or underdeveloped can be largely corrected by suitable choice of paper and manipulation in development of the print.

Agitation is an important factor in the developing procedure and its lack may cause undesirable effects upon the quality of the photographic image. The accumulation of developing by-products near the surface of the emulsion layer causes a general decrease in negative contrast. It also has been shown that in a lack of agitation there is considerable loss of emulsion speed. The necessity for agitation should not be restricted to the phase of development only but should also be used in all other processing operations, e.g., short stop bath, fixation and washing. The formation of air bells on photographic emulsions can be avoided by soaking them in fresh clean water before development. It must be noted that developers after use lose their energy and must be renewed or replenished with fresh solution. If this is not done there is a constant drop in gamma and emulsion speed, causing a loss of detail.

Printing

The end result of a negative is the quality of the final print. Failure to use proper care in printing the negative into a good print will nullify the previous effort taken in producing a well balanced negative. Here again the beginner should restrict himself to the use of a few types of paper until he masters the technique of printing. It is best to use a developer made especially for papers and the instructions supplied by the maker of a specific type of printing paper must be followed.

The methods used for printing are two: contact and projection. Printing papers are supplied in several contrast grades to suit negatives differing in contrast due to variations in exposure and development of the negative. The contrast of printing papers is inherent in the emulsion and the paper surface and can only be controlled within narrow limits by variations in development time or type of developer employed. A paper of suitable contrast must be used to fit the negative and should be exposed for proper density when developed for the recommended time. There is a tendency to over- or underexposure in printing, with resulting prints that are muddy, poor in tone and lacking in contrast. Stains and fogging of the print with loss of detail "flat" of the image also results. Fresh developing solutions should always be employed to avoid brownish stains. Chloride papers, used for contact printing, have a long scale and are comparatively easy to handle. Azo, Velox, and Apex are preferred by many photographers. The "Azo" papers come in six contrasts for proper selection. They produce prints of strong blue-black tones.

Bromide and chlorobromide are enlarging papers, under such trade names as Kodabromide or Velour black, and are designed for projection printing. They are fast papers, require careful handling and, if properly used, yield a long brilliant scale and fine detail. For enlarging, "Velour Black R," which comes in four contrasts, was found to be the most satisfactory. This chlorobromide type of paper gives the greatest range of tones between black and white, resulting in brilliant images with fine detail. Glossy surface papers exhibit a maximum tone range and fineness of detail, heightened when the prints are dried on chromium-plated ferrotype tins. Single weight stock papers are best for quick drying and easy mounting.

Printing by the enlarging method should be used when possible and whole or part of a negative may be enlarged to any required size. This also permits the worker to have full control of light exposure of various parts of the print. Enlargers equipped with condensing lenses produce prints of higher contrast than ones with diffuse illumination. The lens of the enlarger should be of the highest quality and of the proper focal length to cover the plate in order to prevent poor over-all definition or falling off in definition at the corners of the print. The closing of the diaphragm of the enlarging lens does not increase the definition of the image but only controls the amount of light which passes the lens. The image must be carefully focused with the diaphragm wide open at first and then closed. The amount of light that reaches the paper must be of a proportion to permit a controlled exposure time and must not be too short or too long. Stray light sometimes passes the edge of the negative and causes fogging or veiling of the paper. This is prevented by masking the negative and many enlargers are fitted with masks to cut off this light.

In conclusion the success of good photomicrography depends on several factors:

1. Proper objectives, substage condenser, and eyepieces.
2. Type of illumination.
3. Color filter.
4. Characteristics of the photographic emulsion.
5. Exposure time and development of the negative.

The end result of the above will be reflected on the final print. The beginner will do well to follow carefully the instructions given in this chapter and master the simple techniques before expanding his interests to other more difficult procedures used in photomicrography. There are special publications dealing with photomicrography which are recommended to the worker in the bibliography at the end of this volume.

APPENDIX A

LIST OF PRECAUTIONS TO INSURE GOOD GROWTH

The following points have already been discussed, but are listed together to emphasize for the beginner some of the most frequent sources of failure and suggestions for avoiding them.

Bacteria. The first point to consider is whether any bacteria are present. An occasional circumscribed colony in the surrounding medium might be due to dust contamination and does not necessarily interfere with the growth or mean that the materials used were infected, but it would preclude any possibility of transplantation, since this would spread the infecting organisms. This type of culture may be used for experimental fixation and staining.

A cloudy liquefied medium, however, means a definite break in technique, and many such cultures in a series would necessitate the testing of the sterility of all materials involved before using them again.*

Temperature of the Incubator. If no infection is present, one should next check the temperature of the incubator, taking into consideration the possibility of fluctuations during the night. Too high a temperature will kill the tissue, too low will retard growth. A recording thermometer is a valuable safeguard.

Sealing. Was sufficient paraffin applied to coverslip cultures to prevent drying and is it still intact? Are stoppers of tubes and flasks tightly in place?

Condition of the Tissue. If infection, faulty temperature and desiccation are eliminated, the condition of the tissue itself must be considered. Was it torn or injured during preparation, and how soon after death was it obtained? Was it ever exposed to direct sunlight or ever allowed to dry?

Glassware. Is it of poor quality? Was it thoroughly rinsed free of soap, and was the alcohol pure? Remember also that overheating during the sterilizing process may liberate alkali from the glass.

The Medium. Inquiry should be extended to possible errors in the preparation of the various ingredients of the medium.

Tyrode. The Tyrode is most frequently at fault in its tendency to excess alkalinity. This has been discussed in Chapter V. However, the pH may be right and yet the Tyrode imperfect on account of salts which are insufficiently pure or inaccurately weighed. The method of distillation of the water and the cleaning of the filter should also be questioned and other methods of sterilization checked such as possible loss of water in autoclaving with resulting hypertonicity.

---

* See the end of Chapter V.

Extract. The extract need not be suspected of inhibiting growth if it is not contaminated and if it was freshly made from living embryos and with accurately prepared Tyrode. Under these conditions its pH will almost surely be right.

Plasma. The condition of the bird or animal from which the blood was obtained is important. Was it young, healthy, and properly starved before bleeding? Was the pH of the plasma tested? If it is outside the range of 7 to 8, the plasma should be discarded. On account of buffering reactions, adjustments of the pH of plasma are difficult to make and are impermanent.

Appendix B

CONVERSION TABLES

To reduce Fahrenheit (F.) to Centigrade (C.) use the formula: 5/9 (F.° — 32), e.g., 98.6°F. = 5/9 (98.6 — 32) = 37°C.

To reduce Centigrade to Fahrenheit use the formula: 9/5 (C.°) + 32, e.g., 37C.° = 9/5 (37) + 32 = 98.6°F.

EQUIVALENTS

1 inch = 25.4 mm.
1 inch = 2.54 cm.

1 ounce = 28.35 grams

1 minim = .062 cc.
1 fluid dram = 3.697 cc.
1 fluid ounce = 29.57 cc.
1 pint = 473 cc. (0.473 liters)
1 quart = 946 cc. (0.946 liters)

1 mm. = 0.03937 inches
1 cm. = 0.3937 inches
1 meter = 39.37 inches
1 gram = 15.432 grains
1 gram = 0.3527 ounces
1 kilogram = 22 pounds
1 cc. = 16.23 minims
1 cc. = 0.27 fluid drams
1 cc. = 0.033 fluid ounces
1 liter = 1.0567 quarts

Appendix C

SOURCES OF SUPPLY FOR SPECIAL TISSUE CULTURE MATERIALS*

Glassware	Where Obtainable
Ampules—Kimble K. Brand #12011M, 1 ml. capacity, constricted neck	Kimble Glass Co. 10 Rockefeller Plaza, New York, N. Y.
Coverslips—⅞ circles #1, #4010 40 × 40 mm. #2, #4015	Albert A. Henning & Co. 16 East 23rd Street, New York, N. Y.
Columbia staining dishes	Arthur H. Thomas Co. Philadelphia, Pa. or Albert A. Henning & Co.
Depression slides Maximow, 75 × 45 mm. oval depression, 3 × 1 inch round depression, 3 × 1 inch	Albert A. Henning & Co. or Clay-Adams, Inc. 141 E. 25th Street, New York, N. Y.
Flasks—Carrel, 3.5 cm., 5.0 cm.	Albert A. Henning & Co. or Corning Glass Works Corning, New York
—Porter, roller-flask	Karl Shuman 681 Jefferson Ave., Cliffside, N. J.
Pipettes—Pyrex, 1.5 ml. capacity graduated in tenths, inner diameter at tip approx. 2 mm.	Albert A. Henning & Co. or E. Machlett & Co. 220 East 23rd Street, New York, N. Y.
Pipette containers—W. S. 3322	Corning Glass Works
Roller tubes—thin walled, 15 × 150 mm.	Corning Glass Works
Tissue grinder—to specifications	E. Machlett & Co.
Depression slides—optically flat for phase contrast microscopy	Paul Rosenthal 505 Fifth Avenue, New York, N. Y.
Instruments	
Arterial cannula—metal (Porter) A, smallest; B, medium	Harvard Apparatus Co. Back Bay P. O., Boston, Mass.

---

* This is a partial list only of possibly difficult to find items and some of the sources where they may be obtained. It is offered merely as an aid in locating such material.

Cataract knives—all sizes	Albert A. Henning & Co.
Serrefine (blood vessel clamp) 1⅜ inch, recently made by	Albert A. Henning & Co.
listed by	E. F. Mahady & Co., Boston, Mass

MISCELLANEOUS ITEMS	WHERE OBTAINABLE
Filters—Bush positive pressure XA-0277 with STD #33992	Corning Glass Works
Pyrex fritted gas filter GVYYL—31760	Corning Glass Works
Selas filters	Selas Corp. Philadelphia, Pa.
Boerner Centrifugal filter	Arthur H. Thomas Co. Philadelphia, Pa.
Mica coverslips—40 × 40 mm.	Albert A. Henning & Co.
Perforated cellophane	Miss A. J. Hollender, Tissue Culture Assoc., Sloan-Kettering Institute, New York, N. Y.
Rubber nipples Natural latex, 2 cc. capacity 4 cc.	Howe & French, Inc. 99 Broad Street, Boston, Mass. or Albert A. Henning & Co.
Red rubber stoppers—"No Air" #1 for Carrel flasks #2 for roller tubes	Standard Scientific Supply Co. 34 West 4th Street, New York, N. Y.
Romicron microphotometer for determining exposures for photomicrography	Paul Rosenthal 505 Fifth Avenue, New York, N. Y.
CONSTITUENTS OF MEDIA	
Fibrinogen, Fraction I, from bovine plasma	Dr. Leach, Research Division Armour & Co. or Dr. E. J. Cohn, Harvard Medical School, Boston, Mass.
Thrombin, topical, bovine origin	Parke, Davis & Co. Detroit, Mich.
Liquaemin (Heparin sodium)	Organon Inc., Orange, N. J.
Reagent chemicals, C.P. for biological work	Merck & Co. Rahway, N. J.

The following is a list of frequently used components of tissue culture medium now available commercially. These products have all been certified by the test laboratories of the Tissue Culture Association.

PRODUCT	WHERE OBTAINABLE
Fowl plasma (desiccated)	Difco Laboratories, Detroit, Mich.
Chick embryo extract (desiccated)	Difco Laboratories, Detroit, Mich.
Human placental serum (desiccated)	Difco Laboratories, Detroit, Mich.

PRODUCT	WHERE OBTAINABLE
Horse serum (desiccated)	Difco Laboratories, Detroit, Mich.
Distilled H_2O	Difco Laboratories, Detroit, Mich.
Balanced salt solution (Gey's) formula in Chapter V	Difco Laboratories, Detroit, Mich.
Phenol Red. 0.5% in 0.85% NaCl	Difco Laboratories, Detroit, Mich.
Balanced salt solution (Simms) X6, X7, Z	Microbiological Associates, Inc. Flemington, N. J.
Balanced salt solution (Hanks)	Microbiological Associates, Inc.
Serum ultrafiltrate ox, horse, sheep, human	Microbiological Associates, Inc.
Fischer's nutrient V-614	Microbiological Associates, Inc.
Ox serum	Microbiological Associates, Inc.
Sheep serum	Microbiological Associates, Inc.
Human serum (pooled)	Microbiological Associates, Inc.
Triple distilled water	Microbiological Associates, Inc.
Defatted chick embryo extract (desiccated)	Microbiological Associates, Inc.

BIBLIOGRAPHY

Anderson, E. and W. Haymaker, 1936. Elaboration of hormones by pituitary cells growing *in vitro*. *Proc. Soc. Exptl. Biol. Med.* **33,** 313.

Baker, L. E., 1935. Effect of vitamin A on proliferation of fibroblasts. *Proc. Soc. Exptl. Biol. Med.* **33,** 124.

Baker, L. E. and A. Carrel, 1926a. Action on fibroblasts of the protein fraction of embryonic tissue extracts. *J. Exptl. Med.* **44,** 387.

Baker, L. E. and A. Carrel, 1926b. Effect of the amino acids and dialyzable constituents of embryonic tissue juice on the growth of fibroblasts. *J. Exptl. Med.* **44,** 397.

Bang, Frederik B. and G. O. Gey, 1948. A fibrillar structure in rat fibroblasts as seen by electron microscopy. *Proc. Soc. Exptl. Biol. Med.* **69,** 86.

Bland, J. O. W. and R. G. Conti, 1935. The growth and development of psittacosis virus in tissue cultures. *J. Path. Bact.* **40,** 231.

Bodian, D., 1936. A new method for staining nerve fibres and nerve endings in mounted paraffin sections. *Anat. Record* **65,** 89.

Broadhurst, J., Gladys Cameron and V. Saurino, 1938. Measles inclusion bodies in blood and in tissue cultures. *J. Infectious Diseases* **62,** 6.

Broadhurst, J., Gladys Cameron and I. Taylor, 1938. Tissue cultures of human throat inclusion bodies. *J. Infectious Diseases* **62,** 21.

Brues, A. M., E. N. Rathbun and W. E. Cohn, 1944. Metabolism of tissue cultures. II. Estimation of total growth in tissue culture; growth in incomplete media as a "steady state." *J. Cellular Comp. Physiol.* **24,** 155.

Buchsbaum, R., 1948. Individual cells under phase microscopy before and after fixation. *Anat. Record* **102,** 19.

Burrows, M. T., 1910. The cultivation of the tissues of the chick embryo outside the body. *J. Am. Med. Assoc.* **55,** 2057.

Burrows, M. T., 1913. The tissue culture as a physiological method. *Trans. Congr. Am. Phys. Surg.* **9,** 77.

Cameron, Gladys, 1948. Evidence of retention of physiological differentiation in outgrowing sheets in tissue culture. *Anat. Record* **100,** 646.

Cameron, Gladys and R. Chambers, 1937. Neoplasm studies. III. Organization of cells of human tumors in tissue culture. *Am. J. Cancer* **30,** 115.

Cameron, Gladys and R. Chambers, 1938. Direct evidence of function in kidney of an early human fetus. *Am. J. Physiol.* **123,** 482.

Cameron, Gladys, M. J. Kopac and R. Chambers, 1943. Neoplasm studies. IX. The effects in tissue culture of N, N-dimethyl-p-phenylenediamine on rat liver tumors induced by p-dimethylaminoazobenzene. *Cancer Research* **3,** 281.

Caplin, S. M., 1947. Growth and morphology of tobacco tissue cultures *in vitro*. *Botan. Gaz.* **108,** 379.

Carlson, J. G., 1946. Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis. *Biol. Bull.* **90,** 109.

Carpenter, Esther, 1942. Differentiation of chick embryo thyroids in tissue culture. *J. Exptl. Zool.* **89,** 407.

Carpenter, Esther, 1947. A mincing apparatus for the preparation of embryo extract for tissue culture. *Science* **106,** 621.

Carrel, A., 1912. The permanent life of tissues outside of the organism. *J. Exptl. Med.* **15,** 516.

Carrel, A., 1923. A method for the physiological study of tissues *in vitro. J. Exptl. Med.* **38,** 407.

Carrel, A., 1926. Some conditions of the reproduction *in vitro* of the Rous virus. *J. Exptl. Med.* **43,** 647.

Carrel, A. and M. T. Burrows, 1910. Cultivation of adult tissues and organs outside the body. *J. Am. Med. Assoc.* **55,** 1379.

Carrel, A. and A. H. Ebeling, 1921. Age and multiplication of fibroblasts. *J. Exptl. Med.* **34,** 599.

Carrel, A. and A. Ebeling, 1922. Pure cultures of large mononuclear leucocytes. *J. Exptl. Med.* **36,** 365.

Cary, L. R., 1933. Growth of some tissues of Ptychodera bahamensis *in vitro. Carnegie Inst. Wash. Pub.* No. 435, **28,** 195.

Caspe, S. and Gladys Cameron, 1944. Relation of diaphorase to cell growth. *Arch. Biochem.* **5,** 291.

Caspe, S. and Gladys Cameron, 1946. Effect of a respiratory enzyme system and creatine upon the growth of cells *in vitro. J. Cellular Comp. Physiol.* **27,** 43.

Chambers, R. and Gladys Cameron, 1932. Intracellular hydrion concentration studies. VII. The secreting cells of the mesonephros in the chick. *J. Cellular Comp. Physiol.* **2,** 99.

Chambers, R. and Gladys Cameron, 1943. The effect of *l*-ascorbic acid on epithelial sheets in tissue culture. *Am. J. Physiol.* **139,** 21.

Chambers, R. and Gladys Cameron, 1944. Adrenal cortical compounds and *l*-ascorbic acid on secreting kidney tubules in tissue culture. *Am. J. Physiol.* **141,** 138.

Chambers, R. and R. T. Kempton, 1933. Indications of function of the chick mesonephros in tissue culture with phenol red. *J. Cellular Comp. Physiol.* **3,** 131.

Chambers, R. and M. J. Kopac, 1937. Handbook of Microscopical Technique. Edited by C. E. McClung, 2nd edition, Paul Hoeber, Inc., New York, p. 71.

Charipper, H. A. and A. B. Dawson, 1928. Direct division of erythrocytes and the occurrence of erythroplastids in the circulating blood of Necturus. *Anat. Record* **39,** 25.

Coman, D. R. and N. G. Stabler, 1941. An apparatus for roller tube tissue culture. *Science* **94,** 569.

Cornman, I., 1944a. Survival of normal cells in penicillin solutions lethal to malignant cells. *Science* **99,** 247.

Cornman, I., 1944b. A selective lethal effect of penicillin on sarcoma cells growing with normal tissue in roller tube cultures. *J. Gen. Physiol.* **28,** 113.

Cowdry, E. V., 1943. Microscopic Technique in Biology and Medicine. Williams and Wilkins Co., Baltimore.

Cunningham, B. and P. L. Kirk, 1942. Measure of growth in tissue culture. *J. Cellular Comp. Physiol.* **20,** 343.

Dederer, Pauline H., 1921. The behavior of cells in tissue culture of fundulus heteroclitus with special reference to the ectoderm. *Biol. Bull.* **41,** 221.

Des Ligneris, M. J. A., 1936. Tissue culture of long duration in liquid medium. *Arch. exptl. Zellforsch. Gewebezücht.* **18,** 442.

Doljanski, L. and R. S. Hoffman, 1943. Growth activating effect of extract of adult tissue on fibroblast colonies *in vitro;* cultivation for prolonged periods. *Growth* **7,** 67.

Doljanski, L. and H. Werner, 1945. Studies on growth-promoting factor of adult tissue extracts; precipitation with alcohol; action of lipid solvents. *Growth* **9,** 229.

Dubin, I. N., 1947. Bodies suggesting exoerythrocytic forms of plasmodium vivax in tissue culture. *Proc. Soc. Exptl. Biol. Med.* **65,** 154.

Earle, W. R., 1932. A simple press for mincing soft tissue. *Arch. exptl. Zellforsch. Gewebezücht.* **13,** 510.

Earle, W. R., 1934. A technique for the adjustment of oxygen and carbon dioxide tensions, and hydrogen ion concentration, in tissue cultures planted in Carrel flasks. *Arch. f. exper. Zellforsch.* **16,** 118.

Earle, W. R., 1937. A further study of the Walker rat mammary carcinoma No. 256 *in vitro. Arch. exptl. Zellforsch. Gewebezücht.* **20,** 140.

Earle, W. R., 1939. Use of strip-shaped explants in tissue cultures. *Arch. Path.* **27,** 88.

Earle, W. R., 1943. Production of malignancy *in vitro.* IV. The mouse fibroblast cultures and changes seen in living cells. *J. Natl. Cancer Inst.* **4,** 165.

Earle, W. R., 1943. The mouse fibroblast cultures and changes seen in the living cell. *J. Natl. Cancer Inst.* **4,** 165.

Ebeling, A. H., 1921. Measurement of the growth of tissues *in vitro. J. Exptl. Med.* **34,** 231.

Ebeling, A. H., 1922. A ten year old strain of fibroblasts. *J. Exptl. Med.* **35,** 755.

Ebeling, A., 1925. A pure strain of thyroid cells and its characteristics. *J. Exptl. Med.* **41,** 337.

Enders, J. F., 1948. Propagation of Viruses and Rickettsiae in Tissue Cultures. Chap. 5. Viral and Rickettsial Infections of Man. Edited by T. M. Rivers. J. B. Lippincott Co., Philadelphia.

Evans, V. J. and W. R. Earle, 1947. The use of perforated cellophane for the growth of cells in tissue culture. *J. Natl. Cancer Inst.* **8,** 103.

Favata, B. V., 1947. A fibrinogen-thrombin clot useful in tissue culture. *Arch. Path.* **44,** 321.

Fell, H. B., 1939. The origin and developmental mechanics of the avian sternum. *Trans. Roy. Soc. London.* **A229,** 407.

Fell, H. B. and R. Robison, 1929. The growth, development and phosphatase activity of embryonic avian femora and limb buds cultivated *in vitro. Biochem. J.* **23,** 769.

Findlay, G. M., 1928. A note on the cultivation of the virus of fowl-pox. *Brit. J. Exptl. Path.* **9,** 28.

Firor, W. M. and G. O. Gey, 1947. The study of malignant cells with phase difference microscopy. *Ann. Surg.* **125,** 604.

Fischer, A., 1922a. A three months old strain of epithelium. *J. Exptl. Med.* **35,** 367.

Fischer, A., 1922b. A pure strain of cartilage cells *in vitro. J. Exptl. Med.* **36,** 399.

Fischer, A., 1941a. Die Bedeutung der Aminosäuren fur die Gewebezellen *in vitro. Acta Physiol. Scand.* **2,** 144.

Fischer, A., 1941b. The nature of the growth-promoting substances in the embryonic tissue juice. *Acta Physiol. Scand.* **3,** 54.

Fischer, A., 1948a. Nutrition of animal tissue cells. *Scientia* **6,** 170.

Fischer, A., 1948b. Morphological aspects of animal tissue cells in synthetic media. *Acta Anat.* **5,** 57.

Fischer, A., 1949. The application of Soybean inhibitor in tissue cultivation. *Science* **109,** 611.

Fischer, A. and T. Astrup, 1942. Untersuchungen über den Eiweiss-stoffwechsel der Gewebezellen *in vitro. Arch. ges. Physiol.* (Pflügers) **245,** 633.

Fischer, A. and T. Astrup, 1943. Untersuchungen über die chemischen Eigenschaften des tierischen Wuchsstoffes aus Embryonen. *Arch. ges. Physiol. Pflügers* **247,** 34.

Fischer, A., T. Astrup, G. Ehrensvard and V. Oehlenschluger, 1948. Growth of animal tissue cells in artificial media. *Proc. Soc. Exptl. Biol. Med.* **67,** 40.

Francis, T., Jr. and T. P. Magill, 1935. Cultivation of human influenza virus in an artificial medium. *Science* **82,** 353.

Francke, C., 1948. Some observations on the morphological structure of explanted immature ovaries and on "ovariohypophyses." *Acta Neerland. Morphol.* **6,** 1.

Frew, J. G. H., 1928. Technique for the cultivation of insect tissue. *Brit. J. Exptl. Biol.* **6,** 1.

Gaillard, P. J., 1948. Growth, differentiation and function of explants of some endocrine glands. *Symposia Soc. Exptl. Biol., No. II, Growth* 139.

Galston, A. W., 1948. On the physiology of root initiation in excised asparagus stem tips. *Am J. Botany* **35,** 281.

Gatenby, J. B., 1932. A technique for studying growth and movement in explants from Helex Aspersa (snail). *Arch. exptl. Zellforsch. Gewebezücht.* **13,** 665.

Gavrilov, W. and S. Cowey, 1941. Essai de culture *in vitro* de tissus de moustiques et d'intestins de lapins adults infectes. *Ann. humaine et parasitol. comparée* **18,** 180.

Geiling, E. and M. R. Lewis, 1935. Further information regarding the melanophore hormone in the hypophysis cerebri. *Am. J. Physiol.* **113,** 534.

Gemmill, C. L., G. O. Gey and R. Austrian, 1940. The metabolism of tissue cultures of Walker Rat sarcoma 319. *Johns Hopkins Hospital Bull.* **66,** 167.

Gey, G. O., 1933. An improved technic for massive tissue culture. *Am. J. Cancer* **17,** 752.

Gey, G. O. and M. K. Gey, 1936. The maintenance of human normal cells and tumor cells *in vitro*. I. Preliminary report: Cultivation of mesoblastic tumors and normal tissue and notes on method of cultivation. *Am. J. Cancer* **27,** 45.

Gey, G. O., M. K. Gey, F. Inui and H. Vedder, 1944. Penicillin action on strains of normal and cancer cells. A.A.A.S. Res. Conference on Cancer, p. 321.

Gey, G. O., J. H. Hanks and R. Barrett, 1948. Retardation of growth and metabolism of normal and malignant cells during continuous cultivation. *Growth* **12,** 69.

Goldfeder, Anna, 1940. Further studies on effects of irradiation on proliferation and metabolic processes of normal and malignant tissues. IV. Effects produced by different dosage rates of X-ray radiation on the proliferation of various tissues grown *in vitro*. *Radiology* **35,** 210.

Goldfeder, Anna and Gladys Cameron, 1948. Growth in tissue culture of analogous mouse mammary carcinomas and their response to radiation. *Cancer Research* **8,** 465.

Goss, C. M., 1933. Further observations on differentiation of cardiac muscle in tissue cultures. *Arch. exptl. Zellforsch. Gewebezücht.* **14,** 175.

Grand, C. G., 1944. Tissue culture studies of cytoplasmic inclusion bodies in lymph nodes of Hodgkin's disease. *Proc. Soc. Exptl. Biol. Med.* **56,** 229.

Grand, C. G., 1949. Cytoplasmic inclusions and the characteristics of Hodgkin's diseased lymph nodes in tissue culture. *Cancer Research* **9,** 183.

Grand, C. G., and Gladys Cameron, 1948. Tissue culture studies of pigmented melanomas: fish, mouse and human. The Biology of Melanomas. *Spec. Publ. N. Y. Acad. Sci.* **4,** 171.

Grand, C. G., R. Chambers and Gladys Cameron, 1935. Neoplasm studies. I. Cells of melanoma in tissue culture. *Am. J. Cancer* **24,** 36.

Grand, C. G., M. Gordon and Gladys Cameron, 1941. Neoplasm studies. VIII. Cell types in tissue culture of fish melanotic tumors compared with mammalian melanomas. *Cancer Research* **1,** 660.

Hades, H. L., L. T. Webster and G. K. Lavin, 1940. The use of ultra violet light in preparing a non-virulent anti-rabies vaccine. *J. Exptl. Med.* **72,** 437.

Hanks, J. H., 1947. A study of the bacilli in tissue cultures of lepromata in serum media. *Intern. J. Leprosy* **15,** 21.

Hanks, J. H., 1948. The longevity of chick tissue cultures without removal of the medium. *J. Cellular Comp. Physiol.* **31,** 235.

Hanks, J. H., 1949. Calcification of cell cultures in presence of embryo juice and mammalian sera. *Proc. Soc. Exper. Biol. & Med.* **71,** 328.

Harrison, R. G., 1907. Observations on the living developing nerve fibre. *Proc. Soc. Exptl. Biol. Med.* **4,** 140.

Hecke, F., 1931. Die Züchtung des Maul-und Klauensencheerregers. *Zentr. Bakt. Parasitenk. Abt. I* **Ref. 102,** 283.

Herrell, W. E. and D. Heilman, 1943. Tissue culture studies on cytotoxicity of bactericidal agents. III. Cytotoxic and anti-bacterial activity of gramicidin and penicillin; comparison with other germicides. *Am. J. Med. Sci.* **206,** 221.

Hetherington, D. C., 1944. Frozen-dried serum as a medium constituent for tissue cultures. *Proc. Soc. Exptl. Biol. Med.* **57,** 196.

Hildebrandt, A. C., A. J. Riker and B. M. Duggar, 1946. The influence of the composition of the medium on growth *in vitro* of excised tobacco and sunflower tissue cultures. *Am. J. Botany* **33,** 591.

Hoffman, R. S. and L. Doljanski, 1939. Growth activating effect of extracts of adult tissue on fibroblast colonies *in vitro*; experiments with chicken heart extracts. *Growth* **3,** 31.

Hogue, M. J., 1937. Studies of heart muscle in tissue cultures. *Anat. Record* **67,** 521.

Holmes, S. J., 1914. The behavior of the epidermis of amphibians when cultivated outside the body. *J. Exptl. Zool.* **17,** 281.

Holtfreter, J., 1931. Über die Aufzucht isolierter Teile des Amphibienkeimes. *Arch. f. Entwicklungs mech.* **124,** 404.

Hughes, A. F. and M. M. Swann, 1948. Anaphase movements in the living cell. A study with phase contrast and polarized light on chick tissue cultures. *J. Exptl. Biol.* **25,** 45.

Jacoby, F., 1945. The toxicity of sulphadiazine and sulphaguanidine to macrophages *in vitro*. *Brit. J. Exptl. Path.* **26,** 137.

Jacoby, F. and S. J. Darke, 1948. Animal tissue culture with a synthetic medium. *Nature* **161,** 768.

Jacoby, F., P. B. Medawar and E. N. Willmer, 1941. The toxicity of sulphonamide drugs to cells *in vitro*. *Brit. Med. J.* **2,** 149.

Jensen, A. B., 1948. Animal tissue cells in protein-free media. *Nature* **161,** 273.

Kasahara, S., 1935a. On the cultivation *in vitro* of the hypophysis. *Arch. exptl. Zellforsch. Gewebezücht.* **18,** 42.

Kasahara, S., 1935b. Further contributions to the tissue culture of the hypophysis. Re-differentiation of epithelial cells *in vitro*. *Arch. exptl. Zellforsch. Gewebezücht.* **18,** 77.

Kirby, D. B., 1927. The cultivation of lens epithelium *in vitro*. *Arch. Ophthalmol.* **56,** 450.

Kirby, D., K. C. Estey and R. von Weiner, 1933. Effect of changes in medium on cultures of lens epithelium. *Arch. Ophthalmol.* **10,** 37.

Lewis, Margaret R., 1916. Sea water as a medium for tissue cultures. *Anat. Record* **10,** 287.

Lewis, Margaret R., 1928. A simple method for drawing blood from the heart of a fowl. *Arch. exptl. Zellforsch. Gewebezücht.* **7,** 82.

Lewis, Margaret R. and W. H. Lewis, 1911a. The growth of embryonic chicken tissues in artificial media, agar and bouillon. *Johns Hopkins Hospital Bull.* **22,** 126.

Lewis, Margaret R. and W. H. Lewis, 1911b. The cultivation of tissues from chick embryos in solutions of NaCl, $CaCl_2$, KCl and $NaHCO_3$. *Anat. Record* **5,** 277.

Lewis, Margaret R. and W. H. Lewis, 1915. Mitochondria (and other cytoplasmic structures) in tissue culture. *Am. J. Anat.* **17,** 339.

Lewis, W. H., 1926. Cultivation of embryonic heart muscle. *Carnegie Inst. Wash. Pub. No. 363,* 1.

Lewis, W. H., 1935a. Normal and malignant cells. *Science* **81,** 545.

Lewis, W. H., 1935b. Rat malignant cells in roller tube cultures and some results. *Carnegie Inst. Wash. Pub., Contrib. Embryol.* **25,** No. 150, 161.

Lewis, W. H., 1945. Axon growth and regeneration. *Anat. Record* **91,** 25.

Lewis, W. H. and A. G. Richards, 1945. Non-toxicity of DDT on cells in culture. *Science* **102,** 330.

Lewis, Margaret R. and Wm. R. B. Robertson, 1916. The mitochondria and other structures observed by the tissue culture method in the male germ cells of Chorthippus curtipennis scudd. *Biol. Bull.* **30,** 99.

Li, C. P. and T. M. Rivers, 1930. Cultivation of vaccine virus. *J. Exptl. Med.* **52,** 465.

Lillie, R. D., 1948. Histopathologic Technic. The Blakiston Co., Philadelphia.

Loeb, L., 1897. Über die Entstehung von Bindegewebe, Leukozyten und roten Blutkörperchen aus Epithel und über eine Methode isolierte Gewebesteile zu züchten. M. Stern & Co., Chicago.

Loeb, L., 1902. On the growth of epithelium in agar and blood serum in the living body. *J. Med. Research* **8,** 109.

Loosli, C. G., 1936. Section on Outlines of Histological Technique in Methods of Tissue Culture in Vitro. Ralph Buchsbaum, Univ. of Chicago Press, Chicago, Ill.

Maitland, M. C. and H. B. Maitland, 1931. Cultivation of foot-and-mouth disease virus. *J. Comp. Path. Therap.* **44,** 106.

Maximow, A., 1925. Tissue cultures of young mammalian embryos. *Carnegie Inst. Wash. Pub., Contrib. Embryol.* **16,** No. 80, 47.

Morgan, H. R. and R. W. Wiseman, 1946. Growth of psittacosis virus in roller tube tissue culture; use in a vaccine. *J. Infectious Diseases* **79,** 131.

Murray, Margaret R., 1927. Cultivation of planarian tissues *in vitro. J. Exptl. Zool.* **47,** 467.

Murray, Margaret R., 1928. The Ca:K ratio in culture media for P. dorotocephala. *Physiol. Zool.* **1,** 137.

Murray, Margaret R. and A. P. Stout, 1942a. Demonstration of the formation of reticulin by Schwannian tumor cells *in vitro. Am. J. Path.* **18,** 585.

Murray, Margaret R. and A. P. Stout, 1942b. Characteristics of human Schwann cells *in vitro. Anat. Record* **84,** 275.

Murray, Margaret R. and A. P. Stout, 1947a. Distinctive characteristics of the sympathicoblastoma cultivated *in vitro.* A method for prompt diagnosis. *Am. J. Path.* **23,** 429.

Murray, Margaret R. and A. P. Stout, 1947b. Adult human sympathetic ganglion cells cultivated *in vitro. Am. J. Anat.* **80,** 225.

Murray, Raymond G., 1947. Pure cultures of rabbit thymus epithelium. *Am. J. Anat.* **81,** 369.

Osgood, E. E. and I. Brownlee, 1936. Culture of human marrow. Details of a simple method. *J. Am. Med. Assoc.* **108,** 1793.

Osgood, E. E., 1938. Culture of human marrow: An improved apparatus for large scale culture. *Am. J. Med. Sci.* **195,** 141.

Paff, G. H., 1948. Influence of pH on growth of bone in tissue culture. *Proc. Soc. Exptl. Biol. Med.* **68,** 288.

Parker, F., Jr. and R. N. Nye, 1925. Studies on filterable viruses. I. Cultivation of vaccine virus. *Am. J. Path.* **1,** 325.

Parker, R. C., 1936. The cultivation of tissues for prolonged periods in single flasks. *J. Exptl. Med.* **64,** 121.

Parker, R. C. and A. Hollender, 1945a. Propagation of Theiler's GD-VII mouse virus in tissue culture. *Proc. Soc. Exptl. Biol. Med.* **60,** 88.

Parker, R. C. and A. Hollender, 1945b. Propagation of Rabies virus in tissue culture. *Proc. Soc. Exptl. Biol. Med.* **60,** 94.

Pomerat, C. M. and L. Anigstein, 1944. Antireticular immune serum: Its action demonstrated by tissue culture technique. *Science* **100,** 456.

Porter, K. R., A. Claude and E. Fullam, 1945. A study of tissue culture cells by electron microscopy. *J. Exptl. Med.* **81,** 233.

Porter, K. R. and C. V. Hawn, 1947. The culture of tissue cells in clots formed from purified bovine fibrinogen and thrombin. *Proc. Soc. Exptl. Biol. Med.* **65,** 309.

Preston, M. M. E., 1948. Amphibian tissue culture for biophysical research. *Nature* **161,** 203.

Riker, A. J. and Alice E. Gutsche, 1948. The growth of sunflower tissue *in vitro* on synthetic media with various organic and inorganic sources of nitrogen. *Am. J. Botany* **35,** 227.

Rivers, T. M. and S. M. Ward, 1935. Jennerian prophylaxis by means of intradermal injections of culture vaccine virus. *J. Exptl. Med.* **62,** 549.

Robbins, W. J., 1922. Cultivation of excised root tips and stem tips under sterile conditions. *Botan. Gaz.* **73,** 376.

Sabin, A. B. and P. K. Olitsky, 1936. Cultivation of poliomyelitis virus *in vitro* in human embryonic nervous tissue. *Proc. Soc. Exptl. Biol. Med.* **34,** 357.

Sanford, K., W. R. Earle and G. D. Likely, 1948. The growth *in vitro* of single isolated tissue cells. *J. Natl. Cancer Inst.* **9,** 229.

Sano, M. E. and L. W. Smith, 1930. A simplified method for quantitative tissue culture *in vitro. Proc. Soc. Exptl. Biol. Med.* **28,** 282.

Saunders, J. B. deC. M. and W. Haymaker, 1945. Effects of sulfonamides on chick brain tissue cultivated *in vitro. Proc. Soc. Exptl. Biol. Med.* **59,** 306.

Shaw, D. T., L. C. Kingsland and A. M. Brues, 1940. A roller bottle tissue culture system. *Science* **91,** 148.

Simms, H. S., M. S. Parshley and R. B. Pitt, 1947. Fat deposition *in vitro* caused by lipfanogens and opposed by antilipfanogen. *J. Gerontol.* **2,** 205.

Simms, H. S. and M. Sanders, 1942. Use of serum ultrafiltrate in tissue cultures for studying deposition of fat and for propagation of viruses. *Arch. Path.* **33,** 619.

Simms, H. S. and N. P. Stillman, 1937a. Substances affecting adult tissue *in vitro.* I. The stimulating action of trypsin on fresh adult tissue. *J. Gen. Physiol.* **20,** 603.

Simms, H. S. and N. P. Stillman, 1937b. Substances affecting adult tissue *in vitro.* II. A growth inhibitor in adult tissue. *J. Gen. Physiol.* **20,** 621.

Simms, H. S. and N. P. Stillman, 1937c. Substances affecting adult tissue *in vitro.* III. A stimulant (the "A Factor") in serum ultrafiltrate involved in overcoming adult tissue dormancy. *J. Gen. Physiol.* **20,** 649.

Spoerri, Rosette, 1948. A tri-base-dye stain for nerve cells. *Stain Technol.* **23,** 133.

Steinhardt, E., C. Israeli and R. A. Lambert, 1913. Studies on the cultivation of the virus of vaccinia. *J. Infectious Diseases* **13,** 294.

Stone, H. B., J. C. Owings and G. O. Gey, 1934. Transplantation of living grafts of thyroid and parathyroid glands. *Ann. Surg.* **100,** 613.

Strangeways, T. S. P. and H. B. Fell, 1926a. Experimental studies on the differentiation of embryo tissues growing *in vivo* and *in vitro*. I. *Proc. Roy. Soc. London* **B99,** 340.

Strangeways, T. S. P. and H. B. Fell, 1926b. Experimental studies on the differentiation of embryo tissues growing *in vivo* and *in vitro*. II. *Proc. Roy. Soc. London* **B100,** 273.

Theiler, M. and H. H. Smith, 1937. The effect of prolonged cultivation *in vitro* upon the pathogenicity of yellow fever virus. *J. Exptl. Med.* **65,** 767.

Uhlenhuth, E., 1914. The cultivation of the skin epithelium of adult frog Rana pipiens. *J. Exptl. Med.* **20,** 614.

Uhlenhuth, E., 1916. Changes in pigment epithelium and iris pigment cells of Rana pipiens induced by changes in environment. *J. Exptl. Med.* **24,** 689.

Vogelaar, J. P. M. and E. Erlichman, 1933. A feeding solution for cultures of human fibroblasts. *Am. J. Cancer* **18,** 28.

Vogelaar, J. P. M. and E. Erlichman, 1939. Contributions to tissue culture technique. *Am. J. Cancer* **35,** 510.

Webster, L. T. and A. D. Clow, 1937. Propagation of rabies virus in tissue culture. *J. Exptl. Med.* **66,** 125.

Weiss, P., 1934. *In vitro* experiments on the factors determining the course of the outgrowing nerve fibre. *J. Exptl. Zool.* **68,** 393.

Weiss, P. and A. C. Taylor, 1946. The viability of isolated nerve fragments and its modification by methylene blue. *J. Cellular Comp. Physiol.* **27,** 87.

White, P. R., 1942. A Handbook of Plant Tissue Culture. The Jaques Cattell Press, Lancaster, Pa.

White, P. R., 1946. Cultivation of animal tissues *in vitro* in nutrients of precisely known constitution. *Growth* **10,** 231.

Willmer, E. N., 1935. Tissue Culture. Methuen & Co., Ltd., London, pp. 3, 66.

Zernike, F., 1935. Das Phasenkontrastverfahren bei der Mikroskopischen Beobachtung. *Z. techn. Phys.* **16,** 454.

Zernike, F., 1942. Phase contrast, a new method for the microscopic observation of transparent objects. I. *Physica* **9,** 689; 974.

Zinsser, H. and E. B. Schoenbach, 1937. Studies on the physiological conditions prevailing in tissue cultures. *J. Exptl. Med.* **66,** 207.

SUPPLEMENTARY REFERENCES

Some Handbooks and Monographs on Tissue Culture

Bloom, W., 1937. Cellular differentiation and tissue culture. *Physiol. Revs.* **17,** 589.

Buchsbaum, R., 1936. Methods of Tissue Culture *in Vitro*. Univ. of Chicago Press, Chicago, Illinois.

Craciun, E. C., 1931. La Culture des Tissus en biologie experimentelle. Masson et Cie., Paris.

Demuth, F., 1930. Praktikum der Züchtung von Warmblütergewebe *in vitro*. Müller und Steinicke, Munich.

Ephrussi, B., 1932. La Culture des Tissus. Edition Gauthiers Villars, Paris.

Erdmann, Rhoda, 1927. Kaltblütergewebe, in Abderhalden's Handbuch der biologischen Arbeitsmethoden. Urban and Schwarzenberg, Berlin. Abt. 5, Teil 1, Heft 4.

Erdmann, Rhoda, 1930. Praktikum der Gewebepflege oder Explantation besonders Gewebezüchtung. Julius Springer, Berlin.

Fischer, A., 1925. Tissue Culture. Studies in experimental morphology and general physiology of tissue cells *in vitro*. Levin & Munksgaard, Copenhagen.

Fischer, A., 1930. Gewebe-Züchtung. Müller und Steinicke.

Fischer, A., 1946. Biology of Tissue Cells. G. E. Stechert & Co., New York.

Fell, H. B., 1940. The application of tissue culture *in vitro* to embryology. *J. Royal Microscop. Soc.* **60,** 95.

Lewis, W. H. and Lewis, Margaret R., 1924. Behavior of cells in tissue cultures. Cowdry's General Cytology. 2nd edition, Chapter 7, Univ. of Chicago Press, Chicago, Illinois.

Levi, G., 1934. Explantation, besonders die Struktur und die biologischen Eigenschaften der *in vitro* gezüchteten Zellen und Gewebe. Ergeb. Anat. Entwicklungsgeschichte **31,** 125-707.

Parker, R. C., 1938. Methods of Tissue Culture. Paul B. Hoeber, Inc., New York.

Strangeways, T. S. P., 1924. The Technique of Tissue Culture "in vitro." W. Heffer & Sons, Ltd., Cambridge, England.

White, P. R., 1943. A Handbook of Plant Tissue Culture. Jaques Cattell Press, Lancaster, Pa.

Willmer, E. N., 1935. Tissue Culture, the Growth and Differentiation of Normal Tissues in Artificial Media. Methuen & Co., Ltd., London.

Some Handbooks and Monographs on Photomicrography

Allen, R. M., 1941. Photomicrography. D. Van Nostrand, Inc., New York.

Photomicrography. Eastman Kodak Co., Rochester, New York, 1944.

Lester, H. M., 1947. Photo-Lab-Index. Morgan & Lester, New York.

Shillaber, C. P., 1944. Photomicrography in Theory and Practice. John Wiley & Sons, Inc., New York.

Index

A

Adult tissues
  cultures of, 78, 95
  extracts of, 54, 57
  growth of, 2, 84, 87
  preparation of, 66
  use as reagents, 117
Air conditioning, 6, 7
Alcohol-formalin-acetic solution, 132
Anesthesia
  of cold-blooded animals, 52
  of fowl, 44, 45
  of rats, 50
Animal board
  description of, 26
  use of, 44, 48
Antibiotics
  penicillin, 56, 66
  streptomycin, 66
Anti-coagulants
  heparin, 49, 50, 78
  sodium citrate, 50
Autoclave, 7
AzoRubin S, 128

B

Bacteria
  control of, 31, 56
  result of faulty technique, 80
  tests for, 5, 39, 40
Bakelite square
  description of, 24
  use of, 80
Balance
  analytical, 9
  care of, 10
  trip scale, 8
Bleeding
  from carotid artery, 43-47
  cold-blooded animals, 51, 52
  fowl, 42
  from heart, 48, 50
Bleeding—(*Continued*)
  mammals, 49, 50
  from wing, 47, 48
Blocks, wooden, for tubes, 25
Board for culture table
  description of, 23, 24
  use of, 64, 80, 81
Bone marrow
  cultivation of, 74
  extract of, 58
Bouin's fixative, 132
Brilliant Cresyl Blue, 128
Brom Cresol Purple, 38
Buffy coat, 47
  extract of, 58
  preparation of from blood, 69

C

Camera for photomicrography, 153
  Bausch & Lomb, 155
  Compur shutter, 155
  Graflex for, 154
  35 mm. cameras, 156, 157
  Micro Ibso attacshment, 156
Camera lucida, 118
Candling of eggs, 55
Cannula, arterial, 20
  use of, 46, 51
Carnoy's fluid, 132
Carotid bleeding
  instruments for, 19
  technique for, 43-47
Carrel flasks, 83-89
  cleansing of, 17
  drum for rinsing, 17, 18
  microscopic examination of, 88
  preparation of cultures in, 85-87
  subculturing, 88
  use of, 85, 88, 116
Cartilage
  appearance of, 104
  development of, 99

Cartilage—(*Continued*)
  pure strain of, 71
Cellophane, 14
  perforated, 14, 89, 95
Cell support
  agar, 2, 41, 59
  fibrinogen, 61, 78, 88
  gelatin, 41
  mechanical, 14, 41, 76
  plasma, 2, 41, 72, 78, 86, 92
Cell types, description of
  connective tissue, 102-104
    cartilage, 104
    fibrocytes, 102-104
    non-cellular fibers, 104
  epithelioid cells, 101, 110
  epithelium, 104-106
    ciliated, 106
    columnar, 105
    cuboidal, 105
    glandular, 105, 106
    pigmented, 106
    squamous, 105
  multinucleated, 100, 101, 106, 110, 112, 115
  muscle, 106-107
    cardiac, 106
    skeletal, 107
    smooth, 107
  nervous elements, 107-108
    brain, 108
    nerve cells, 108
    neuroglia, 108
    spinal cord, 108
  wandering cells, 99-102
    granulocytes, 99, 100
    histiocytes, 100, 101
    lymphocytes, 101
    macrophages, 100, 101
    monocytes, 100
Centrifuge, 8
Clasmatosis, 113
Cleaning mixture, 13
Cold-blooded organisms, 2
  bleeding of, 51, 52
  cultivation of tissues from, 2, 79, 80
  explants of, 99
  extracts of, 56
  physiological solutions for, 40
  in roller tubes, 95
Cold-blooded organisms—(*Continued*)
  sterility of, 67, 68
  temperature for, 74, 80
Color filters, 151
  cooling liquid, 153
  exposure time, 152
  gelatin, 151
  glass, 152
  inconel, 153
  interference, 152
  neutral, 152
  use of, 152
Color photomicrography, 159
  color temperature, 159
  exposure time, 159
Columbia staining dish
  for fixation and staining, 130
  for vital staining, 129
  for washing cultures, 83, 84
Contaminations
  common sources, 31
  common types in cultures, 80
  prevention of, 31, 32, 58
  testing for, 5, 39, 40
Coverslips
  blocks for drying, 14, 15
  Cellophane, 14
  mica, 14, 83
  quality of, 13
  use of, 75-84
  washing of, 14, 15
Cresol red, 38
Culture containers, 73
  Carrel flasks, 12, 85
  depression slides, 11, 72, 73, 82, 83
  Erlenmeyer flasks, 89
  Earle's flask, 85
  Grand's flask, 85
  roller bottles, 94
  roller flasks, 94
  roller tubes, 12, 92
Culture cubicle, 6
Culture room, 5-7
  cleansing of, 31
  equipment of, 7
  lighting, 5
  ventilation of, 5
Cultures
  chick tissues, 78, 87, 95
  cold-blooded tissues, 79, 95

Cultures—(*Continued*)
  contaminations of, 31, 80, 166
  cytological study of, 72, 76, 116
  on double coverslips, 73, 81-84, 117
  examination of, 80, 88, 93, 117, 118
  experimental use of, 116-121
  fixation of, 130, 131
  in flasks, 72, 92-95, 116
  of insects, 79, 80
  instruments for, 19
  mammalian tissues, 78, 82, 95
  measurement of growth, 118, 119
  medium for—*See media*
  mixed cultures, 63, 102
  neoplastic tissues, 82, 83
  position of, 77, 83
  preparation of table for making, 64
  pure strains, 63, 68-71
  requisites for success, 1, 3, 5, 31, 32, 34, 35
  in roller tubes, 72, 92-95, 116
  on single coverslips, 72, 76-81
  sources of failure, 166, 167
  staining of, 131
  subcultures, 80, 88, 93
  vital staining of, 128, 129
Cytological study of cells, 1, 3, 72, 101, 116
  in comparative studies, 100, 101, 117, 118
Cytoplasmic inclusions, 101
  development of, 90
  vital staining of, 128

D

Darkroom, 161
Depression slides, 11, 18, 72, 73, 82, 83
Desiccated media
  where obtainable, 170, 171
Developing plates and films, 162
  agitation, 163
  contrast, 163
  fixing and washing, 163
  gamma, 162
  time and tmperature, 163
Differentiation
  of bone, 99
  of cartilage, 99
  of epithelium, 96, 104, 105
  in explants, 96-99
Differentiation—(*Continued*)
  methods to enhance, 4, 72, 82, 85, 86
  of muscle, 96, 106, 107
  temperature to enhance, 74
Double coverslip method, 81-84

E

Eggs, fertile
  candling of, 55
  incubation of, 54
  incubator for, 9
Endotoxins, 35
Environment, 1, 3, 74
  aeration, 1
  hydrogen ion concentration, 34, 35, 74
  nutriment, 1
  temperature, 1, 74
Eosin, 133, 136
Epithelium
  appearance of, 104, 105
  differentiation of, 96, 97, 105, 106, 114
  pure strains of, 67-71
Equipment
  care of, 10
  permanent, 7
Erlenmeyer flasks
  cultures in, 89
  for propagation of viruses, 90, 91
Experimental procedures, 116-119
Explant, study of
  bone, 99
  cardiac muscle, 98
  cartilage, 99
  chorio-allantoic membrane, 96
  cold-blooded forms, 99
  kidney, 96, 97
  primitive streak, 99
Extract—*See tissue extracts*

F

Fibrinogen
  for flask cultures, 88, 89
  pH of, 62
  preparation of, 61
  for roller flask cultures, 94
  for slide cultures, 79
  storage of, 61
Fibroblasts (fibrocytes)
  appearance of, 102, 103
  Carrel's "old strain," 3, 69

Fibroblasts—(*Continued*)
  curtailment of, 4, 72, 81, 82
  pure strain, 69
  stimulation of, 72, 75, 81
Filters
  Berkefeld, 38
  Boerner centrifugal, 53
  Bush positive pressure, 38
  Seitz, 38, 53
  Selas, 38
Fixation, 130
  fluid, 131
  precautions, 130
  vapor, 131
Fixatives, 131
  precautions in use of, 130
  suggested list of, 131, 132
Fluid medium
  advantages of, 76
  fixation of cultures in, 131
  limitations of, 76
  maintenance of cultures in, 77
  preparation of cultures in, 76, 77
"Flying" coverslips for micromanipulation, 124
Formalin-Ringer solution, 132
Formalin vapor, 131

G

Gaseous medium, 87, 89, 93
Gelatin
  ash-free, 68, 79, 80
  use in nutrient for cold-blooded tissues, 95
Giemsa stain, 133-134
Glassware
  cleansing of, 12-18
  packing of, 18
  quality of, 11
  sterilization of, 32
  suggested list of, 11, 12
Granulocytes, 47
  appearance of, 99-102
  extract of, 58
  preparation of from blood, 69
  in spleen cultures, 100
Growth
  measurement of, 2, 118, 119
  order of, 102, 104, 113
Growth—(*Continued*)
  requirements for, 1, 3, 60, 63, 74, 76
  stimulation of, 54, 75
Guinea pigs
  carotid bleeding, 50
  heart puncture, 50

H

Heart puncture
  of cold-blooded animals, 52
  of fowls, 48
  of rats, 50
Hematoxylin, 131
  Delafield's, 131
  Harris', 131
Heparin
  for chicken plasma, 47
  for mammalian plasma, 49, 50, 78, 79
  for roller tube cultures, 93
Histological methods, 130-137
Hydrogen ion—*See pH*

I

Illumination, 144
  centering of, 146
  low voltage-high amperage, 145
  ribbon filament, 145
  zirconium lamp, 145
Illumination for micromanipulation, 125
Incubator
  for cultures, 8
  for eggs, 9
  room, 7
  temperature of, 54, 74
Insects
  cultures of, 79, 80
  physiological solutions for, 40
  preparation of tissues of, 68
Instruments
  arrangement of, 22, 23
  sharpening of, 23
  sterilization of, 32
  suggested list of, 19
Invertebrate tissues
  cultures of, 79, 80
  physiological solutions for, 40
  preparation of tissues, 67, 68
Iodine vapor, 131

J

Janus Black, 129
Janus Green, 128, 129

K

Kelvin (°K) color temperature, 159
Kidney tubules
  cultivation of, 96, 97, 98
  differentiation of, 105
  teasing of, 66
Knives
  care of, 23
  cataract, 19, 21
  packing of, 22, 23
  removable blades, 19, 21, 64
  sharpening of, 23
  sterilization of, 32

L

Leucocytes—*See granulocytes*
Liquefaction of medium, 83, 84, 87
  due to contamination, 166
  inhibition of, 62
  by malignant tissues, 83, 87, 93, 109, 110, 114

M

Magnification, determination of, 143
Mammalian tissues
  cultures of, 78, 82
  preparation of, 66, 67
Mammals
  bleeding of, 49, 50
  plasma of, 41, 44, 78, 79
  preparation of tissues of, 66
  serum of, 53
  tissue extracts of, 56
Measurement of growth
  by increase in area, 118
  by mitotic activity, 119
  by physiological activity, 119
  by weight, 119
Media
  for chick tissues, 78
  coagulated, 6, 78, 79
  for cold-blooded animals, 79, 80
  fluid, 3, 71, 74, 76, 89, 90, 93
  gaseous, 87, 89, 93
  heterologous, 86, 95
  for invertebrates, 79, 80
  of known composition, 59, 60, 61
Media—(*Continued*)
  lyophilized, 3, 41, 49, 57
  for mammalian tissues, 78
  nutrient fluid for flask and tube cultures, 86, 87, 90, 92
  nutrient fluid for cold-blooded tissues, 95
  pH of, 62, 86, 87
Methylene Blue, 128
Micromanipulation, 122-127
  of tissue cultures, 122
Microneedles, 126
Microscopes
  care of, 10
  dark field, 115
  electron, 115
  inverted, 85, 138
  objectives of, 8
  phase contrast, 115
Mitochondria
  fixation of, 131
  in living cells, 108, 115
  staining in fixed cultures, 134
  vital staining of, 128, 129
Mitosis, 2, 101, 103, 106
  multipolar, 109
  in neoplastic cells, 109, 114
Mitotic counts, 119
Mixed cultures, 63, 102
Monocytes, pure strain of, 69
Muscle
  appearance of, 106, 107
  cardiac, 98, 106, 107
  contraction of, 98, 107
  cross striations in, 98, 107
  differentiation of, 96, 106, 107
  irritability of fibers, 107
  myofibrillae, 107
  skeletal, 98, 107
  smooth, 98, 107

N

Needles
  dissection, 21, 22
  hypodermic, 22
  surgical, 21, 22
Nervous tissues
  appearance of, 108
  brain, 108
  growth of, 2, 107, 108

Nervous tissues—(*Continued*)
  neurofibrils, 107, 108
  neuroglia, 108
  spinal cord, 107, 108
  staining of, 134, 135
Neutral Red
  application of, 129
  preservation of in fixed cultures, 136
  use of, 128

O

Objectives
  achromatic, 142
  apochromatic, 142
  depth of focus, 141
  fluorite objectives, 142
  magnification, 143
  numerical aperture, 141
  water immersion objective, 143
  working distance, 141
Ocular micrometer, 118
Oculars, eyepieces, 143
  Huyghenian, 143
  periplane, 143
  magnification, 143
Operating sheet, 28
Optical equipment, care of, 144
Orange G, 128
Osmic vapor, 131
Osmotic pressure, 34, 37

P

Paraffin
  for sealing cultures, 30, 76
  for coating tubes, 42, 43
Penicillin, 56, 66
Perforated cellophane
  cleansing of, 89
  use of, 89, 95
pH
  adjustments of, 38, 39, 87
  experimental alterations of, 117
  of fibrinogen-thrombin medium, 62, 89
  importance of, 34, 35
  indicator for, 86
  optimum for chick tissues, 35
    mammalian tissues, 35, 68
  of plasma, 167
  testing of, 38
  of Tyrode, 35, 37
Phase contrast microscopy, 147
Phenol red
  secretion of, 96
  sterilization of, 35
  use of, 35, 86, 117
Photoelectric exposure meters, 160
Photomicrography, 138-165
  cameras, 153
  color, 159
  color filters, 151
  dark field, 140
  exposure time, 159
  phase contrast, 149
  plates and films, 157
Physiological solutions
  formulae
    for warm-blooded animals, 40
    for cold-blooded animals, 40
  pH of, 35, 36
  preparation of, 34-39
  sterilization of, 36, 37, 38
  use of, 34
Pigment
  in chromatophores, 107
  development of, 99, 105
  in epithelial cells, 106
  in fibrocytes, 111
  in macrophages, 111, 112, 113
  in melanoblasts, 112, 113
  in melanophores, 113
  in melanomas (fish, human, mouse), 111-113
Pipettes
  bent tip, 16
  capillary, 16
  cleansing of, 17
  coarse, 16
  containers for, 17
  graduated, 17
  making of, 16
  rack for, 24
Plant tissues, 2
Plasma
  chicken, obtaining of, 41-47
  heparinized, 78, 79
  lyophilized, 41, 49
  mammalian, 41, 44, 78, 79
  storing of, 41, 47
Plates and films for photomicrography, 157
  antihalation, 157

Plates and films for photomicrography—(*Continued*)
  characteristics of, 158
  color films, 159
  developing, 162
  exposure time, 159
  orthochromatic plates, 158
  panchromatic plates, 158
  printing, 164
  process plates and films, 158
  resolving power, 157
  superspeed 35 mm. film, 159
Position of cultures, 77, 83
Preparation room, equipment of, 7
Printing
  bromide papers, 164
  chloride papers, 164
  chlorobromide papers, 164
  contrast, 164
  enlarging, 164
  methods of, 164
Projectoscope, 118
Proliferation, 3, 4, 72, 75, 81, 86, 89
Pure strains of cells, 63
  methods for obtaining, 68-71
    cartilage, 71
    crystalline lens, 70
    endocrine gland epithelium, 71
    fibroblasts, 69
    iris epithelium, 70
    malignant cells, 70
    monocytes, 69
    renal epithelium, 70
    thymus epithelium, 70
    thyroid epithelium, 71
  use as reagents, 117

R

Racks
  for Carrel flasks, 28, 88
  for pipettes, 24
  for roller tubes, 27
  for slide cultures, 25, 26
Records, keeping of, 117, 118
  sample records, 119-121
Refrigerator, 9
Ringer solution
  formulae, 40
  sterilization of, 32
  use of, 37, 43
Roller tubes
  for cold-blooded tissues, 95
  cultures in, 92-95
  drum for, 26, 93
  microscopic examination of, 93
  racks for, 27
  subcultures, 93, 94
  uses of, 95, 116
Roller tube drum
  description of, 26
  use of, 92, 95
Rubber nipples
  cleansing of, 29
  sterilization of, 29
Rubber stoppers
  cleansing of, 29
  no-air stoppers, 29, 86
  solid rubber, 29, 86

S

Sand bags
  description of, 26
  use of, 44
Sea water
  formula, 40
  use in media, 76, 79
Sealing of cultures, 77
Sectioning, 136, 137
Serrefine
  description of, 20
  use of, 45, 46, 51, 52
Serum
  chicken, 52
  filtration of, 53
  heating of, 53
  heterologous, 53
  horse, 53, 78
  human placental cord, 53, 78
  mammalian, 53
  ultrafiltrates of, 60, 61, 78
Silver impregnation
  Bodian's method, 135-136
  Polak's modification of Rio-Hortega's method, 134
Solid medium
  preparation of cultures in, 78
    on coverslips, 78-84
    in flasks, 85-91
    in roller tubes, 92-95

Spatula, platinum, 21
  use of, 86
Special supplies
  sources of, 169, 170, 171
Spoerri's tribasic stain
  for nerve cells, 134-135
Staining procedures, 133-136
  with Giemsa, 133, 134
  for mitochondria, 134
  for nerve cells, 134, 135
  routine, 133
Sterilization
  by antibiotics, 56, 66
  by chemicals, 56, 66, 67, 68
  by filtration, 37, 38, 53
  of glassware, 32
  by heat (Arnold, autoclave, dry heat), 32, 33
  of instruments, 32
  of invertebrate tissues, 67, 68
  by irradiation, 67
  of tadpoles, fish fry, etc., 67
  of tissue extracts, 58
  of Tyrode solution, 36-38
Sterilizers
  autoclave, 8
  dry heat, 8
Subcultures
  frequency of, 80, 84, 87, 93
  method for coverslips, 80, 81, 84
    cellophane, 89
    flasks, 88
    roller tubes, 93, 94
Substage condenser, 139
  Abbe, 139
  achromatic, 139
  aplanatic, 139
  centering condenser, 139
  dark field, 140
  numerical aperture, 140
Sulfonamide compounds
  use of, 66, 67

T

Table board, 23, 64
  position of, 24
Temperature
  for cold-blooded tissues, 74, 80
  conversion scale, 168
  of culture incubators, 8, 74
Temperature—(*Continued*)
  of egg incubators, 54
  of refrigerator, 9
  for sterilization, 32
  for viruses, 90
Thrombin
  pH of, 62
  preparation of, 61
  storage of, 61, 62
  use of, 62, 79, 88, 94
Tissue extracts, 54-58
  adult, 57
  analysis of, 59, 60
  bone marrow, 58
  chick embryo in small amounts, 55, 56
  inflamed tissue, 58
  large amounts, 57
  lyophilized, 57
  mammalian, 56
  mechanical devices for making, 57
  purpose of, 3, 54
  sterilization of, 58
  storage of, 58
  substitutes for, 59, 60
  tadpole, 56
  turbidity of, 54, 56
Tissues, 63
  plant, 2
  preparation of adult, 66
    cold-blooded, 67
    embryonic, 64, 66
    invertebrate, 67, 68
    neoplastic, 67
  storage of, 68
Trypsin
  inhibitor, 62
  preparation of solution, 94
  use of, 94
Tumors
  carcinomas, 113
  dissection of, 67
  lymphomas, 109, 110
  melanomas, 111
  pure strains of, 70
  sarcomas, 111
Tyrode solution
  adjustment of pH, 36, 38, 39
  formula for, 34
  pH of, 35, 36, 37, 38
  preparation of, 35-37

Tyrode solution—(*Continued*)
  sterilization by filtration, 37, 38
    steam, 36

U

Ultrafiltrates (serum), 60, 61, 78
Ultraviolet radiation, 5, 58, 67

V

Virus
  on coverslips, 90
  cultivation of, 61, 90
  in flasks, 90
  specificity of, 91
Vital staining, 128, 129

W

Wandering cells, 99-102
  granulocytes, 69, 99, 100, 101, 102
  histiocytes, 99, 100, 101
  Kupfer cells, 101
  lymphocytes, 99, 100, 101, 102, 109, 110, 115
  macrophages, 99, 100, 101, 102, 111,113
  monocytes, 69, 100
  reticular cells, 101
  from spleen, 100

Z

Zenker's fluid, 132
Zenker-formol (Helly's fluid), 132

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
LIBRARY

1.10.80 5/5/97
8/89
C. F. T. R. I. LIBRARY, MYSORE.

A

CHECKED
2008

VERIFIED
2013

CFTRI-MYSORE
982
Tissue culture t..